Isolation of sensory neurons in vitro and generation of a conditional inducible transgenic mouse line against nerve growth factor (NGF) receptors to investigate the role of NGF in pain.

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Isolation of sensory neurons *in vitro* and generation of a conditional inducible transgenic mouse line against nerve growth factor (NGF) receptors to investigate the role of NGF in pain.

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Abstract

Nerve growth factor (NGF) is a known factor in the development of persistent pain, a condition which affects approx. 20% of the UK population. Current therapeutics available for pain exhibit limited effectiveness and therefore better targeted and more effective therapeutics are essential. Clinical trials using anti-NGF have been successful in consistently alleviating pain of patients suffering from chronic pain however the main mechanism of action of NGF is unknown. The main aim of this PhD was to investigate the role for TrkA and p75 receptors for NGF in the development of persistent pain, specifically targeted to primary sensory afferents. The first aim was to determine whether NGF acts directly or indirectly (via other cell types reported to express receptors for NGF) on sensory neurons in the development of pain. To address this we optimised and characterised a protocol for purifying sensory neuronal cultures from dorsal root ganglia (DRG) using magnetic assisted cell sorting (MACS), a method we found to reliably produce viable 95% pure neuronal cultures. The second major question surrounding the mechanism of NGF is whether NGF acts mainly through the TrkA or the p75 receptor, or do the two receptors work in synergy. In order to address this we used two different methods. First, we chose to use an approach using viral vectors, both lenti and adeno-associated virus, to introduce Cre recombinase into DRG neurons with floxed regions of the *NTRK1* and *NGFR* genes to knockout expression of TrkA or p75 respectively. Secondly, we bred a new transgenic mouse lines for conditional knockout of TrkA or p75 under the control of tamoxifen by crossing the above mentioned floxed mouse lines with an *Advillin* CreERT2 transgenic mouse, where Cre activity is limited to sensory and sympathetic neurons.

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Abbreviations

AAV – adeno-associated virus

β-III tub – β-III tubulin

CI – confidence interval

CM- conditional medium

DAPI - 4',6-diamidino-2-phenylindole

DEPC - diethyl pyrocarbonate

DRG – dorsal root ganglion

FC- fold change

FD – found dead

GFR – growth factor reduced

GS – gluatamine sythetase

IB4 – isolectin B4

ICC – immunocytochemistry

IENF – intraepidermal nerve fibres

IHC – immunohistochemistry

i.p. -intraperitoneal

IT – intrathecal

KO – knockout

MACS – magnetic-activated cell sorting

MOI – multiplicity of infection

NGF – nerve growth factor

n.s. – not significant

PLL – poly-L-lysine

p75 – low affinity nerve growth factor receptor

RIN – RNA integrity number

RT- room temperature

SGC – satellite glial cells

TMX – tamoxifen

TrkA- tropomyosin receptor kinase A

Chapter 1: General Introduction

Pain according to the definition provided by the international association for the study of pain (IASP) is “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (IASP 2017). For the majority of people pain is a protective mechanism which can be described as nociceptive, inflammatory or neuropathic (Woolf 2010). Following injury the site of injury becomes inflamed and hypersensitive to non-noxious stimuli thereby triggering a drive to protect the injury site from further damage, thus allowing the injury to heal (Dubin and Patapoutian 2010; Woolf and Ma 2007). However, in some cases, following repair of the injury, pain persists and becomes chronic, the reasons for which are unclear. Pain itself can also be described as an emotional response driven by prior experiences (Smith and Lewin 2009). In this way two people with similar injuries can experience different levels of pain. Environmental factors impacting on a person’s emotional state can also play a role. For example, a study performed using soldiers from world war two found that even patients with severe injuries such as penetrating wounds of the torso or abdomen or extensive soft tissue injuries, refused to take more morphine stating that they felt either little or no pain. This lack of pain was attributed to the emotional turmoil associated with conditions on the fighting front line where the relief of being away from the fighting was enough to override the pain from injuries (Beecher 1946). Therefore, pain can be described as a complex state.

Generally, painful stimuli are detected by sensory neurons that innervate peripheral tissues. Two thirds of sensory neurons are known as nociceptors. The term nociceptor, first described by Sherrington, describes a population of neurons that respond to high threshold painful thermal, mechanical and chemical stimuli as an early pain warning system (Dubin and

Patapoutian 2010; Woolf and Ma 2007). Following the induction of a painful stimuli nociceptors project into the dorsal horn of the spinal cord, where the information is integrated and passed up the spinal cord via projection neurons into the brainstem, where the signal is passed to cortical regions of the brain, processed, and pain is perceived (Kuner 2010; Kelleher, Tewari, and McMahon 2016) (**Fig 1.1**). During the acute phase of pain, peripheral and central sensitisation of the pain pathway occurs, a process in which neurotrophins and other inflammatory mediators play a role. However, in some cases following the resolution of injury, pain becomes chronic.

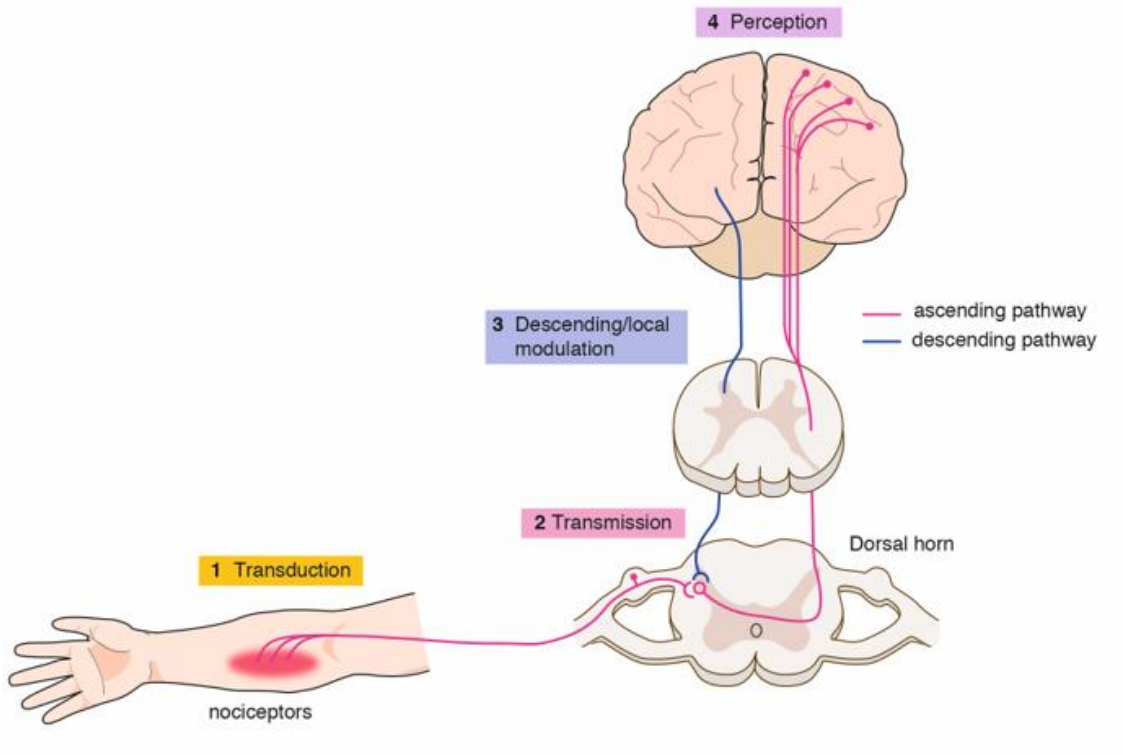


Figure 1.1 Schematic outlining the major anatomical pathways involved in pain processing. *Noxious stimulus is detected in the periphery and transduced into an action potential in a nociceptive neuron. Signal is then transmitted to the dorsal horn of the spinal cord where it may undergo descending or local modulation. Finally, it is transmitted up through ascending pathways of the spinal cord to the brain where it is perceived as pain (taken from Kelleher et al, 2016).*

The prevalence of chronic pain is a worldwide issue with an estimated 6-8% of the UK population alone predicted to suffer from neuropathic pain (Hall et al. 2013; Bouhassira et al. 2008). Worldwide, estimates of the prevalence of all types of chronic pain have risen by 20% (van Hecke, Torrance, and Smith 2013; Breivik et al. 2006) which results in a significant economic burden (The national acamedies press 2011) since treatment is expensive. Chronic pain additionally has a devastating impact on the quality of life in affected individuals. Current treatments for pain are unsuitable for long term use with some analgesic treatments such as opioids having negative side effects including addiction (Z. Y. Wang et al. 2015). However, there are currently a number of phase II and III clinical trials in place to develop novel therapeutics (Rice et al. 2014; Schwertner et al. 2013; M. T. Brown et al. 2012; Ford 2012) including anti-NGF monoclonal antibodies. Despite success of such alternative therapeutics, a number of side effects have been reported (Hertz and Fields 2012) leading to a need for further research in the field. We therefore investigate in this study the detailed role of NGF signalling in pain.

1.1 NGF, TrkA and p75

Nerve growth factor (NGF) is a member of the neurotrophin (NT) family of growth factors (GFs) which also include brain derived neurotrophin factor (BDNF), neurotrophin-3 and 4/5 (NT-3 and NT-4/5). It was first discovered in 1948 by Elmer Bueker, confirmed by Rita Levi-Montalcini (Cohen, Levi-Montalcini, and Hamburger 1954; Levi-montalcini 1987) and then later isolated by Stanley Cohen in 1960 from the submandibular gland in mice (Cohen 1960). NGF was the first member of the NT family to be positively identified, with BDNF and NT-3 isolated subsequently. Over the past 60 years our understanding of this molecule and its role in disease has advanced significantly.

NGF signals via two receptors, the high affinity TrkA receptor and a lower affinity p75 neurotrophin receptor (p75).

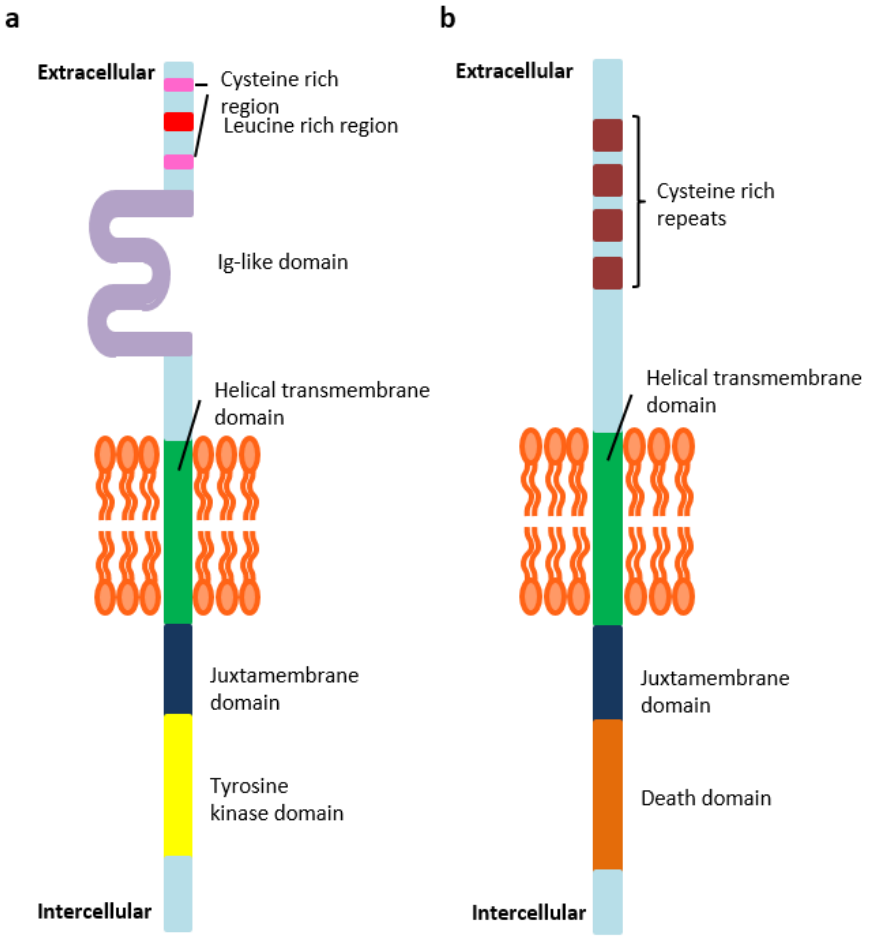


Figure 1.2 Schematic showing the structure of the NGF receptors TrkA and p75 a- *Overview of TrkA receptor and* ***b-*** *overview of p75 receptor.*

TrkA is a member of the tropomyosin family of tyrosine receptor kinases, encoded on chromosome 3 and consisting of 17 exons. It is a transmembrane protein with a highly conserved tyrosine kinase domain in the intracellular region, a helical transmembrane domain and a leucine/cysteine rich extracellular domain containing an IgG-C2 domain (Chao, Hempstead, and Barbara 1995) (**Fig 1.2a**). Trk receptors have a high specificity for a particular

member of the NT family where TrkA is selective for NGF, (with some activation by NT-3). TrkB is selective for BDNF and NT-4/5 and TrkC is selective for NT-3. In the presence of NGF the TrkA receptor monomers dimerise and the intracellular part of the receptor becomes autophosphorylated which leads to the activation of a number of growth and survival downstream signalling pathways including MAPK, AKT and PLCγ (Pezet and McMahon 2006; Kiris et al. 2014; Wiesmann et al. 1999). Phosphorylation of TrkA at Y490 residue permits interaction of TrkA with Shc which in turn activates the MAPK and Ras pathway via Grb-SOS (Capsoni et al., 2011). Y785 phosphorylation however has been shown to facilitate the binding of proteins containing the SH2 domain such as PI3K (Capsoni et al. 2011), CHK1 and PLCγ. Interaction of TrkA with CRK (adaptor protein) has also been reported to activate ERK signalling (W J Friedman and Greene 1999) (**Fig 1.3**).

P75 is a member of the tumour necrosis factor receptor 1 (TNFR1) superfamily and is described as a promiscuous receptor as it is capable of interacting with all members of the NT family (Capsoni and Cattaneo 2006; K. F. Lee et al. 1992). It is encoded on chromosome 11 and contains 6 exons. Like the Trk receptors, p75 is also a transmembrane protein consisting of a helical transmembrane domain. However, the extracellular domain contains four cysteine rich repeat regions and there is a highly conserved unique intracellular domain which includes a death domain region (Chao, Hempstead, and Barbara 1995) (**Fig 1.2b**). Therefore, signalling of NGF via the p75 has been described to activate apoptotic pathways and exert its effect via ERK, JNK and NF-κB via activation of the IL-1 receptor associated kinase (IRAK). However, when co-expressed with TrkA it can promote growth (Meeker and Williams 2014; He and Garcia 2004; W J Friedman and Greene 1999; Mamidipudi, Li, and Wooten 2002) (**Fig 1.3**).

The distinction of NGF signalling via TrkA for growth and survival and via p75 for activation of apoptotic pathways is a simplified view of the signalling pathways involved. Further

investigation and greater understanding of NGF signalling has thus far uncovered a more complex signalling process.

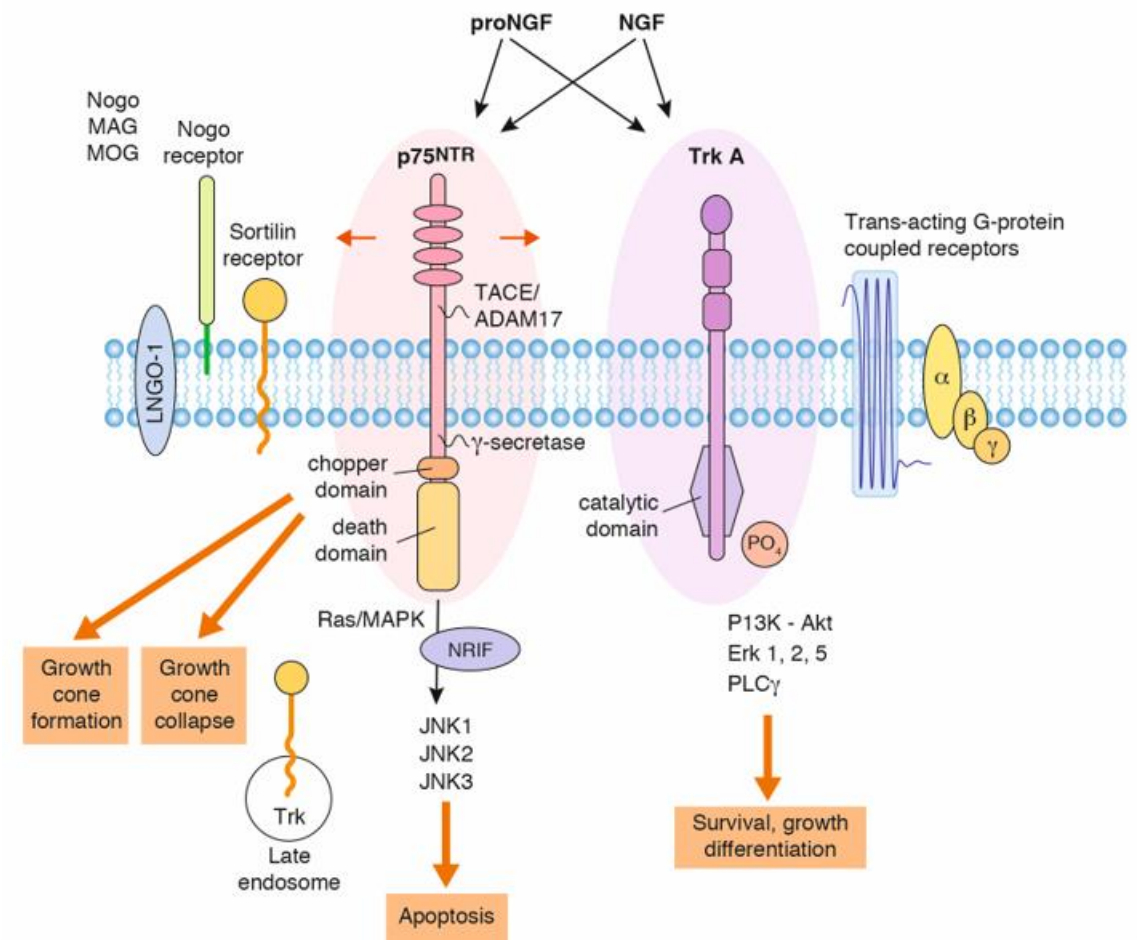


Figure 1.3 NGF exerts its biological effects via TrkA and p75. *NGF signalling via TrkA activates the PI3K-Akt, ERK and PLCγ signalling pathways. TrkA is capable of interacting with p75 and there is some evidence of transactivation of TrkA from G-protein coupled receptors. Activation of p75 signals apoptosis through the JNK pathway, however, in the presence of TrkA p75 can activate survival pathways. P75 is capable of interactions with the sortilin, which can act as a co-receptor for proNGF signalling through p75. In addition, there is some evidence that p75 is capable of interacting with the Nogo receptor (taken from Kelleher et al, 2016).*

NGF is transcribed as a pro-form (proNGF). Following cleavage of the pre NGF signal (pre-proNGF) on entry into the endoplasmic reticulum, proNGF is stored in the Golgi body. This proNGF is then cleaved further by furin to release mature NGF. This mature NGF is stored in vesicles and transported to the nerve terminals while the pro domain of NGF is recycled (D'Onofrio et al. 2011). It has been shown that both the proNGF and mature NGF can be released by cells, particularly following injury, where the pro-form appears to be a selective agonist for p75 (Hempstead 2009; Bruno and Cuello 2006). In addition, a number of other receptors have been identified which modulate NGF signalling. Sortilin for example, a receptor mainly associated with endosomes can control the trafficking of receptors such as TrkA to the cell surface, but in addition, has also been shown to interact with the p75 as a co-receptor for proNGF signalling (Capsoni and Cattaneo 2006; Meeker and Williams 2014). Other studies suggest that the Nogo receptor interacts with the p75 receptor and is able to modulate its signalling, or that activation of G-protein coupled receptors can transactivate TrkA in the absence of NGF (Meeker and Williams 2014). Finally, despite the two receptors controlling opposing signalling pathways there is evidence that TrkA and p75 can interact and modulate downstream signalling (Meeker and Williams 2014). There is evidence that cells co-expressing TrkA and p75 are more specific for NGF and bind NGF with a faster association rate (Chao, Hempstead, and Barbara 1995; Canossa et al. 1996). Hence, NGF signalling is complex, and depends on the presence of both NGF forms and the pattern of different receptors. What is clear however is that many of the biological actions of NGF depend on signalling through both NGF receptors (a Patapoutian and Reichardt 2001) (**Fig 1.3**).

In the adult nervous system, TrkA is expressed on peptidergic C fibres, sympathetic post-ganglionic neurons and small populations of CNS neurons (Usoskin et al. 2014; Thakur et al. 2014; Richner et al. 2014). There is also evidence that TrkA is expressed in the cortex and hippocampal regions of the brain (Capsoni and Cattaneo 2006). In addition, multiple immune

cells are believed to express TrkA including mast cells, macrophages, satellite glial cells (SGC), T and B cells (Aloe et al. 2012; Chan et al. 2004). However, expression of TrkA on immune cells is mostly not supported by recent RNA sequencing studies (Gosselin et al. 2014; Lavin et al. 2014; Mo et al. 2015).

P75 has a more widespread expression pattern, it is co-expressed with TrkA on peptidergic nociceptors but is also present on other sensory neurons including some non-peptidergic neurons, and in moderate amounts on other cells types, including sympathetic post-ganglionic neurons, neutrophil granulocytes, macrophages (Richner et al. 2014; Aloe et al. 2012) and Schwann cells surrounding sensory axons bundles of C fibres (Ebenezer et al. 2007; Aloe et al. 2012) . Surprisingly, there is little expression in the CNS and, as with TrkA, expression on immune cells is not well supported in recent genome wide sequencing studies (Gosselin et al. 2014; Lavin et al. 2014; Mo et al. 2015).

1.2 Role of NGF in development

NGF is essential for the development and survival of sensory and sympathetic neurons (Patel et al. 2003; Delree et al. 1989). Knockout of NGF in mice showed that despite survival *in utero*, knockouts do not survive past weaning with a maximum lifespan of 28 days. Compared to littermates, NGF null mice display developmental delays in hair growth and ptosis and a marked insensitivity to noxious stimuli. In addition, they exhibit a severe loss of cells in the superior cervical ganglia (SCG) and a 70% loss of neurons in the dorsal root ganglia (DRG), specifically small diameter neurons (Crowley et al. 1994). TrkA knockout mice show a similar phenotype to the NGF knockout mice. As with the NGF knockout mice, TrkA knockouts are viable *in utero* surviving to a maximum of 55 days post-natal. Sensory defects as measured by a failure to detect deep pinpricks in whisker pads and rear paws and thermal hyposensitivity are apparent by post-natal day 10 (P10) and at 3-4 weeks of age there are indications of self-

mutilation (Smeyne et al. 1994). These mice also show a loss of neurons in the SCG and a 70-90% loss of DRG neurons, preferentially small diameter neurons (Smeyne et al. 1994). In contrast, mice lacking the p75 are born physically normal and are fertile. Mice are observed to exhibit a loss of epidermal nerve fibres including those positive for calcitonin gene-related peptide (CGRP) (K. F. Lee et al. 1992; Bergmann et al. 1997) and have a deficit in thermal sensitivity (K. F. Lee et al. 1992) and mechanical sensitivity (Bergmann et al. 1997) with some evidence of self-mutilation in hind paws by 4 months of age (K. F. Lee et al. 1992). Although there is no indication of any effect in SCG (K. F. Lee et al. 1992), DRGs appear noticeably smaller however there is no significant loss in a particular population of neurons (Bergmann et al. 1997). Overall, this suggests that NGF signalling via the TrkA and p75 receptors plays a dominant role in development.

Evidence from human studies further suggests a role for NGF in development. Hereditary sensory and autonomic neuropathy (HSAN) IV and V patients exhibit insensitivity to pain as a result of an improper nervous system development. HSAN IV (also known as CIPA – chronic insensitivity to pain with anhydrosis) is an autosomal recessive disease which arises from a mutation in the *NTRK1* gene encoding the TrkA receptor. It was first described in 1996 when testing of multiple families of CIPA patients showed mutations resulting in a frameshift or an alternate splice site in exons 1-3 (Yasuhiro Indo et al. 1996). Since then multiple mutations have been discovered across the 17 exons of TrkA (for review see (Y Indo 2012)). As previously described, NGF is a key regulator during the development of the sensory and sympathetic nervous system and in maintenance of the cholinergic neurons in the basal forebrain (Capsoni and Cattaneo 2006). As a result patients with HSAN IV show symptoms of insensitivity to pain which can result in self-mutilation and serious injuries such as fractures and mental retardation (Yasuhiro Indo et al. 1996) in addition to other symptoms including anhydrosis (inability to sweat) and some indication of an immune phenotype (Beigelman et al. 2009). HSAN V is also

an autosomal recessive disease where patients exhibit insensitivity to pain without anhydrosis or mental retardation as a result of a mutation in the *NGFB* gene encoding NGF (Capsoni et al. 2011). The R100W mutation in NGFβ in HSAN V has been labelled as a "painless" NGF mutation (insensitivity to pain) which results in an inability for cleavage of proNGF to mature NGF and a decreased ability for this proNGF mutant to bind to p75. Interestingly, in a preclinical model, behavioural tests using a similar mutation, R100E, showed a decreased sensitivity to pain compared to wild type NGF treated animals (Capsoni et al. 2011). Investigation of common signalling pathways showed that R1000W and R100E mutations resulted in a loss of phosphorylation of TrkA residues Y490 and Y785 (Capsoni et al. 2011) which suggests that TrkA downstream signalling plays an important role in the development of hyperalgesia. However, a mutation in the *NGFB* gene has recently been reported in a Bedouin family which results in a frameshift in the *NGFB* gene. These patients exhibit signs of a HSAN IV phenotype despite not carrying a mutation in the *NTRK1* gene (Carvalho et al. 2011).

The effects observed in global knockout rodent models and HSAN IV and V patients are both linked to mutations in the NGF signalling pathway (Yasuhiro Indo et al. 1996; Einarsdottir et al. 2004; K. F. Lee et al. 1992; Bergmann et al. 1997; Crowley et al. 1994; Smeyne et al. 1994). The chronic insensitivity to pain in these genetic diseases is the consequence of deficient NGF signalling early in development where sensory and sympathetic neurons are NGF dependent (Crowley et al., 1994). NGF secreted from peripheral tissue binds to the peripheral terminals of axons branching out from the DRG which is then retrogradely transported back to the cell soma, as shown in experiments using labelled NGF. Furthermore, these experiments showed that following axotomy, neurons underwent apoptosis as a result of reduced NGF signalling from the peripheral terminals (Levi-montalcini, 1987). Therefore, in NGF or TrkA knockout mice or human patients with HSAN IV or V, the failure of NGF to either be secreted in the periphery or bind to TrkA receptors results in apoptosis of peptidergic C fibres, and thus a

hyposensitivity to mechanical and thermal noxious stimuli arises. Since this effect is limited to TrkA expressing C fibres all other DRG sensory neurons develop normally, therefore, there is no loss in proprioception for example which is controlled by large diameter fibres (Hudspith, Siddall, and Munglani 2006).

1.3 Changing role of NGF postnatal

The role of NGF in development changes abruptly in the post-natal animal. It has been described that from P3 to P14 there is a downregulation in TrkA expression from 80% of DRG neurons to 40-50% of neurons, which correlates with an increase in the expression of Isolectin B4 (IB4) positive neurons – a marker of non-peptidergic C fibres (Bennett et al. 1996). This change in NGF dependence and differentiation to peptidergic/non-peptidergic C fibres from early developmental events hinges on *Runx1* expression which acts as a molecular switch and is only expressed in non-petidergic fibres (Lallemend and Ernfors 2012; Wainger et al. 2014; Blanchard et al. 2014). Fully differentiated neurons which are NGF independent (for survival) are established by P14 in rodents.

In the post-natal brain, NGF has been implicated in Alzheimer's disease (AD). Although it is not essential in early development (Crowley et al. 1994) of cholinergic neurons it has been well established to have an essential role in the maintenance of these neurons in the basal forebrain through retrograde transport of NGF through them from the cortex and hippocampus to the basal forebrain, a process which is disrupted in AD leading to the apoptosis of cholinergic neurons. Studies in AD11 transgenic mice (a model of AD) showed intranasal application of NGF rescued loss of cholinergic neurons and cognitive defects. Furthermore in AD12 mice (a model of AD) where NGF is neutralised and p75 is knocked out there is absence of β-amyloid deposits or plagues suggesting that there is a complex role for NGF in AD (for review see (Capsoni and Cattaneo 2006)).

1.4 Functional role of NGF in the adult nociceptive system

In the adult the most well described role for NGF is in the development of persistent pain. This is supported by a number of animal studies using pharmacological methods to targeting the NGF/TrkA pathway. A number of peripheral cells have been shown to secrete NGF in response to injury including immune cells, fibroblasts, neurons, smooth muscle cells and epithelial cells (Richner et al. 2014). Andreev *et al* showed that injection of NGF subcutaneously into the hind-paw of rats at doses as low as 250ng could elicit a sustained thermal hyperalgesia that lasted for several hours (Andreev et al. 1995). This was confirmed by Lewin *et al*, 1993, who showed that injection of exogenous NGF into the paw resulted in hyperalgesia as seen by a reduced threshold to mechanical and thermal stimuli (Lewin, Ritter, and Mendell 1993). Furthermore, it has been reported that treatment of mice or rats with anti-NGF or a TrkA blocker results in an attenuation of thermal and mechanical allodynia in a wide range of animal models of persistent pain (Bennett et al. 1998; McMahon et al. 1995; Woolf et al. 1994; Lewin, Ritter, and Mendell 1993). Woolf *et al*, 1994, tested an anti-NGF antibody in rats injected with complete Freud's adjuvant (CFA), an inflammatory model which induces mechanical and thermal hyperalgesia. Compared to CFA treated control animals, animals treated with anti-NGF monoclonal antibody showed a significant decrease in thermal and mechanical hyperalgesia following CFA treatment (Woolf et al. 1994). McMahon *et al*, 1995, used intra-plantar pumps in the hind paws of rats to deliver a TrkA-IgG fusion molecule which blocks signalling of NGF through the TrkA receptor. They observed that rats treated with TrkA-IgG and capsaicin, which induces inflammatory pain, exhibited reduced thermal hypersensitivity compared to animals treated with capsaicin alone (McMahon et al. 1995). In regards to human diseases NGF has been linked to a number of different persistent pain conditions including osteoarthritis (OA), lower back pain (LBP), diabetic peripheral neuropathy (DPN), bladder pain syndrome (BPS), bone cancer pain and endometriosis (Pecchi et al. 2014; Bannwarth and Kostine 2014; Aloe et

al. 2012; Capsoni and Cattaneo 2006; McCaffrey et al. 2014; Alvarez and Levine 2014; Jiang, Liu, and Kuo 2014).

NGF plays an important role in the development and maintenance of chronic pain in adult animals (**Fig 1.4**). Firstly, NGF has a role in the development of peripheral sensitivity. Following peripheral injury or inflammation, there is recruitment of immune cells to the injury site to which the body responds by secreting multiple factors including growth factors (e.g. NGF), cytokines and chemokines as part of an "inflammatory soup". Upon nerve injury neurons immediately release chemokine ligand 1 (CXCL1) and CCL2 which recruit early inflammatory responders – the resident macrophages. Once activated these resident macrophages secrete pro-inflammatory mediators such as NGF, cytokines and chemokines at the site of injury (Barouch et al. 2001; Zhuang et al. 2007). Neutrophil granulocytes attracted by NGF and CXCL1 migrate into the site and secrete further inflammatory mediators (Perkins and Tracey 2000) which in combination with macrophages result in the migration of further macrophages and monocytes (immature macrophages activated to become mature macrophages) to the injury site. Schwann cells secreting NGF and GDNF (glial derived neurotrophin factor) act to sensitise neurons which results in secretion of pro-inflammatory mediators, such as substance P (O'Connor et al. 2004), CGRP, bradykinin and nitric oxide (NO) which vasodilates blood vessels resulting in swelling and oedema. In addition to Schwann cells releasing NGF to sensitise neurons, in inflammation Schwann cells can activate resident macrophages, neutrophil granulocytes and mast cells stimulating further release of pro-inflammatory mediators such as prostaglandin and TNFα (Tofaris et al. 2002). TNFα bound to macrophages further signals the release of pro-inflammatory mediators via the MAPK pathway. NGF bound to macrophages can stimulate further release of TNFα. Altogether, this results in the injury site being bathed in an "inflammatory soup" of NGF, cytokines and chemokines with positive feedback loops in place to maintain secretion of pro-inflammatory mediators from multiple sources

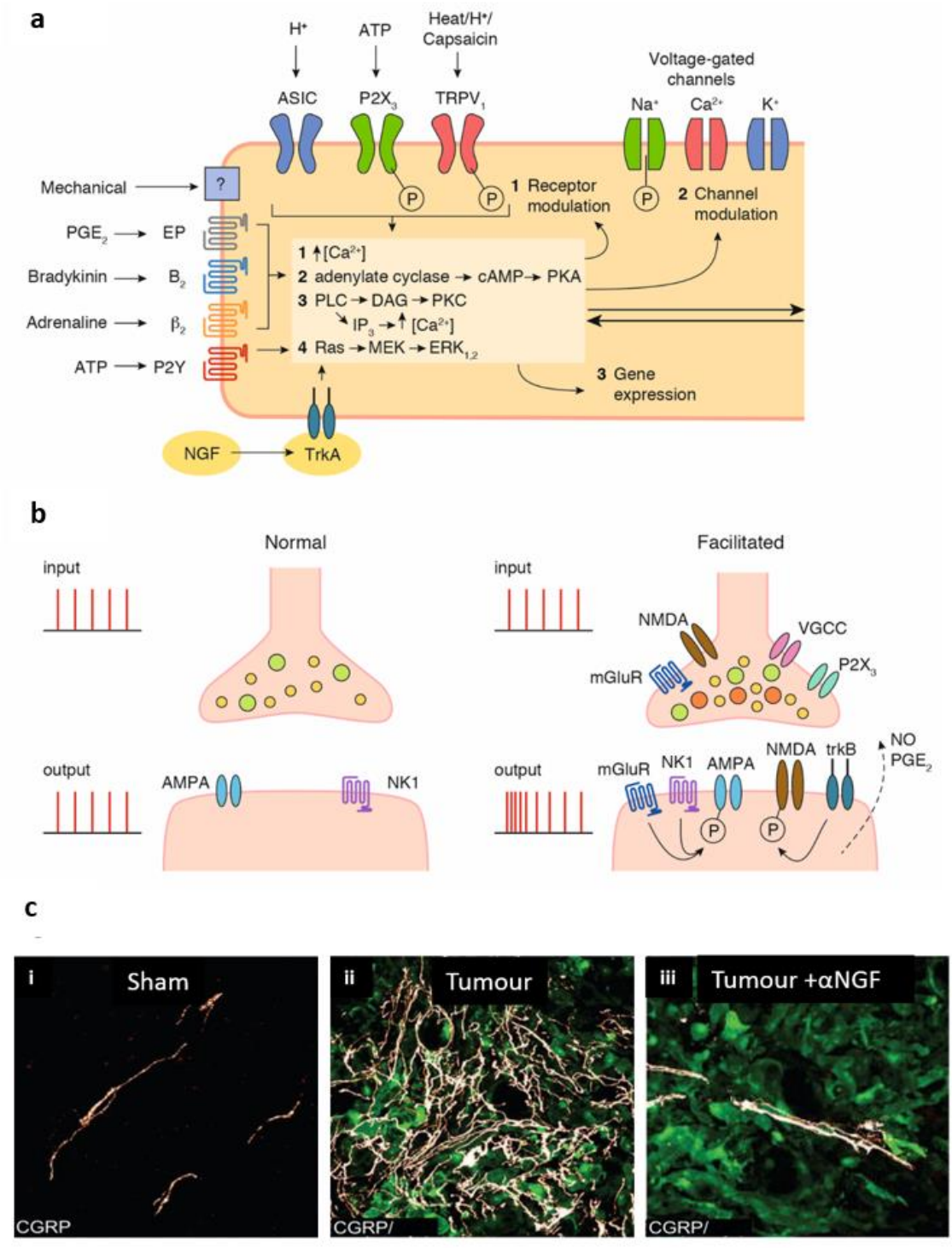


Figure 1.4 Mechanisms of NGF in persistent pain – peripheral sensitisation, central sensitisation and hyper-innervation a- *Schematic of peripheral sensitisation. Injury or inflammation results in the secretion of an "inflammatory soup" which includes factors such as*

NGF, bradykinin and PGE_2 which bind to receptors on peripheral terminals of nociceptors. This results in various processes including receptor modulation, channel modulation and gene expression which all contribute to the process of peripheral sensitisation. ***b****- Schematic of central sensitisation. Noxious stimuli can also be amplified by processes within the CNS. The pharmacology is best understood at a spinal level, where the NMDA receptor plays a pivotal role. This form of modulation is often referred to as central sensitisation.* ***c****- Evidence of nociceptor sprouting in some models of persistent pain. This data shows sections of bone immunostained for CGRP+ sensory nerve fibres in (i) normal mice and (ii) mice with tumours implanted into intramedullary space, and (iii) mice with tumours but treated with anti-NGF. Note the NGF-dependent increase in innervation in this model. However, following anti-NGF treatment in tumour implanted mice hyper-innervation was attenuated (Jimenez-Andrade et al, 2011) (taken from Kelleher et al, 2016).*

including microglia (Clark et al. 2007). Neurons alone have receptors for substance P, TNFα, prostaglandin E2 (PGE2), bradykinin and NGF which all function to sensitise the neuron via the insertion of TRPVI channels into peripheral terminals (A. Patapoutian, Tate, and Woolf 2009). Binding of NGF, and other sensitising compound present in the "inflammatory soup", (Bradykinin, PGE2, TNFα and endothelin to name a few), to receptors on peripheral nociceptors induces rapid sensitisation of the nociceptive response (A. Patapoutian, Tate, and Woolf 2009; Dubin and Patapoutian 2010). NGF (unlike Bradykinin or other sensitising molecules) is also retrograde transported to the cell soma. Increased NGF in the soma results in a phenotypic switch and induces rapid transcription of many pain related genes including TRPV1, which then signals for insertion of this capsaicin sensitive ion channel into the peripheral terminal, thus increasing the sensitivity of the neuron to depolarisation and the increased firing of action potentials associated with nociceptive pain (A. Patapoutian, Tate, and

Woolf 2009; Basbaum et al. 2009). This has been confirmed *in vitro* where NGF has been shown to be important in sensitisation of DRG neurons. Neurons were desensitised following repeated exposure to capsaicin to activate TRPV1 ion channel, however, following treatment of neurons with NGF, they were found to be sensitised and responsive to further exposure to capsaicin (Bonnington and McNaughton 2003). Other pain related genes, such as BDNF, are packed into dense core vesicles and released in response to activity from central processes on nociceptors, contributing to central sensitisation.

Intrathecal injection of NGF has been shown to increase the expression of BDNF in TrkA positive DRG neurons to almost 85% neurons expressing BDNF (compared to 21% in controls), compared to a minimal increase in BDNF expression in TrkB or C positive DRG neurons (Kerr et al. 1999; Michael et al. 1997). In addition, intrathecal NGF results in an increase in the number of axons expressing BDNF in the spinal cord (Michael et al. 1997; Kerr et al. 1999), projecting into laminae I and II from dorsal roots, which also appear to co-express CGRP, another typical marker of TrkA positive neurons (Michael et al. 1997). Further to this, BDNF has been shown to enhance the effect of NMDA receptor on central sensitisation in a model where rats were treated with NGF prior to injection with a TrkB-IgG antibody, after which a reduction in the second phase response to the formalin test was observed, which is also linked to the process of central sensitisation (Kerr et al. 1999).

Another potential mechanism is that NGF causes pain by promoting sprouting of nociceptive fibres in peripheral tissues. NGF has a well-established role in promoting growth of sensory and sympathetic neurons *in vitro* and *in vivo*. In several pain states, for example bone cancer pain, there is an apparent effect for NGF to promote the sprouting of axon terminals of nociceptive neurons resulting in a hyper-innervation of peripheral tissue. It is unclear what functional consequence this has on the nociceptors, but it could result in an increased responsiveness of nociceptors to stimulation and thus an increase in pain. In bone cancer pain

for example sprouting of CGPR positive fibres can be blocked by early treatment with anti-NGF (Jimenez-Andrade et al. 2011). In addition, anti-NGF treatment in a mouse model of bone cancer pain resulted in an attenuated guarding and flinching behaviour in anti-NGF treated mice compared tumour only controls (Sevcik et al. 2005; Jimenez-Andrade et al. 2011; McCaffrey et al. 2014). This was accompanied by a reduction in markers of peripheral sensitisation – ATF-3 and CD-68 positive macrophages in the DRG – and central sensitisation – dynorphin-IR and c-fos in deep laminae of the spinal cord (Sevcik et al. 2005).

Overall, treatment with anti-NGF compounds showed an attenuation of pain related behaviours in accordance with a reduction in peripheral and central sensitisation markers and reversal of hyper-innervation of peripheral tissues suggesting that NGF plays a diverse role in the adult nociceptive system.

1.5 Therapeutic potential of anti-NGF treatment in experimental and clinical studies

Given the range of biological effects of NGF on the nociceptive system, it is not surprising that multiple studies have explored the importance of this factor as a pain mediator in a variety of experimental pain models. These studies began in the 1990's and are still being performed today. The early preclinical studies focussed on inflammatory models induced for instance by carrageenan or CFA (e.g. (Woolf et al. 1994; McMahon et al. 1995). Over the years the range of models tested has increased dramatically and one of the striking features of this literature is the consistency of anti-NGF effects. There is good preclinical evidence for targeting the NGF signalling as an effect analgesic in a number of pain states including acute burn injury, UV inflammation, arthritis and bone cancer to name a few (for review see (Pezet and McMahon 2006)).

Following these findings in preclinical models a number of anti-NGF therapies developed for the clinic have progressed into phase III clinical trials, mainly in osteoarthritis (OA) patients. Multiple anti-NGF monoclonal antibodies are currently in phase III trials including tanezumab (Pfizer), fasinumab (Regeneron) and fulranumab (Janssen and Regeneron). Typically, anti-NGF treatment in clinical trials is administered as part of a combined therapeutic approach with a non-steroidal anti-inflammatory drug (NSAID), the current recommended treatment for OA patients. However, due to an increase in the number of side effects reported, including cases of rapidly accelerated OA (RPOA) and osteonecrosis in both affected and unaffected joints of OA patients clinical trials were put on hold in 2010 by the FDA, and re-evaluated in 2012 (Hertz and Fields 2012; Holmes 2012; Chang et al. 2016). As of March 2015 the hold on clinical trials was lifted on most of the OA trials under the condition of better screening for adverse effects including the autonomic nervous system (Mullard 2015). The incidence of rapidly progressing OA seems to be associated with the combined use of anti-NGF and NSAID treatment so the new trials will focus on anti-NGF monotherapies. However, there was a success in a recent clinical trial where tanezumab and NSAID such as diclofenac was combined. This therapy resulted in a reduction in pain in OA patients with no adverse side effects observed (Balanescu et al. 2014).

Osteonecrosis in joints following treatment could be explained by an increase in weight bearing on affected joints following pain relief however in a number of cases osteonecrosis was also seen in non-weight bearing joints and while weight bearing may be a factor in accelerating degeneration, it may not be the underlying cause (Seidel and Lane 2012). Fasinumab, developed by Regeneron, is also a monoclonal antibody specific for NGF extensively tested in OA patients. Treatment with this drug resulted in a 50% reduction in pain as measured by the WOMAC score measuring joint pain, stiffness and function. As success in therapeutics is measured by an improvement of 30% or more, anti-NGF therapeutics have

been declared effective in these clinical trials. Side effects from Fasinumab ranged from mild to moderate with increasing dosage. The most severe symptoms included joint pain and swelling predominately (Tiseo et al. 2014).

Despite side effects, results from clinical trials have shown a robust reduction in pain compared to placebo in patients in a variety of conditions. Tanezumab has been most widely tested, showing a striking dose dependent effect on reducing pain in OA, with a reduction in pain scores from 20-50% with increasing doses (Lane et al. 2010). This striking effect has been repeated in a number of other subsequent trials. In addition to OA, there have also been beneficial effects of anti-NGF observed in lower back pain (LBP), diabetic peripheral neuropathy (DPN) and bladder pain syndrome in animal models and clinical trials (Bannwarth and Kostine 2014; Capsoni and Cattaneo 2006; Aloe et al. 2012). This includes fulranumab treatment in patients with DPN, which showed a dose dependent alleviation of pain despite early termination of the phase II clinical trial in response to the FDA hold (H. Wang et al. 2014). A meta-analysis of anti-NGF treatment in LBP patients indicated that anti-NGF has a beneficial role in alleviation of pain and functional recovery (mainly tanezumab), although after screening over a thousand studies, only 4 randomised controlled trials were analysed, which further implicates a need for deeper research into the effects of anti-NGF (Leite et al. 2014).

There have also been unsuccessful clinical trials with anti-NGF agents. These include chronic pancreatitis, endometriosis and painful intervertebral discs (for review see (Pezet and McMahon 2006)). Of course, it is not known if these negative findings are the result of patient heterogeneity, poor trial design or due to NGF not being an important pain mediator in these conditions. The range of effect sizes observed in different anti-NGF trials suggests that other important pain mediators beyond NGF contribute to clinical pain states. Further clinical trials will be necessary to clarify the importance of NGF in different pain states and diseases.

Overall, both animal models and clinical trials show anti-NGF to be a highly effective as an analgesic therapeutic (Bannwarth and Kostine 2014). It is not yet a licenced product, and the adverse effects reported may frustrate full development of anti-NGF. However, since there are multiple opportunities to interfere with NGF signalling it is likely that some of these will be explored for potential analgesic effects, hopefully eliciting less side effects.

1.6 Current questions in the NGF field

Despite an extensive amount of research into functional and pharmacological effects of NGF there are still a number of unanswered questions that need to be addressed.

Although it has been established that there is a role for NGF in pain through a number of mechanisms, it is still unclear what the relative importance of these are. As described above, various cells in the PNS and CNS express TrkA and/or p75 and secrete NGF and/or other sensitising components of the "inflammatory soup". Therefore, it is reasonable to question whether NGF sensitisation of sensory neurons is a direct effect of NGF on the neurons themselves, or if it is an indirect effect via another cell type. In addition, there is some debate as to how NGF signals through its two different receptors. From a simplified view, NGF signalling via TrkA promotes survival and growth and signalling through p75 promotes apoptosis. However, almost 60 years after the discovery of NGF, the picture became more and more complicated with more potential contributions and subtle alterations to the signalling pathway coming from a number of different sources as described above (chapter 1.1). It is possible that either TrkA or p75 has an overpowering effect or there could be subtle modulation of signalling between the two receptors. Finally, the contribution of proNGF versus the mature NGF form in the NGF signalling pathway is unclear.

It has been well established that OA patients treated with anti-NGF have a beneficial effect in regards of pain above that of the current pain management options. However, despite

multiple clinical trials the effectiveness and mechanism of anti-NGF is still undergoing testing due to a number of unexplained side effects. Questions such as what is causing these underlying adverse side effects and the suggested impact on the autonomic nervous system require further investigation. It is also not known why anti-NGF treatments are so effective. There are a number of other components of the "inflammatory soup" that could be a useful target for chronic pain therapies, however, they are as yet undiscovered, in the early testing phases, and might not have as much potential as anti-NGF therapies. The effect might be explained with a multi-action effect on peripheral and central sensitisation and, in the case of bone cancer pain, in some cases reversal of hyper-innervation of tissue?

In conclusion, over 60 years of research has shown that NGF is essential in development of sensory and sympathetic neurons and nociceptive pain in the adult organism. Animal models and more recently clinical trials have shown that treatment with anti-NGF is highly effective in the alleviation of pain, specifically in OA, over and above currently prescribed treatments. If the side effect profile of anti-NGF can be overcome or mitigated, it is likely that this treatment could become an important addition to analgesic armoury for patient use.

1.7 Aims of the study

As described above there is great potential for the use of anti-NGF therapies in pain however there are a number of unanswered questions surrounding the role of NGF in pain. In this study we aimed to address two of these questions. First, we investigated whether NGF signals directly onto neurons, or indirectly via non-neuronal cells. We did so by optimising and characterising a method for purifying neuronal cultures from adult DRG using magnetic assisted cell sorting (MACS). Second, we aimed to investigate whether one or both of the NGF receptors, TrkA and p75, play a role in the development of hyperalgesia. To address this, we used two different approaches to specifically knockout expression of either NGF receptor in

the adult mouse using the Cre-LoxP system. The first approach involves the use of viral vectors including both lenti and adeno-associated viruses to deliver Cre into the DRG both in culture and in the animal. The second approach involves the breeding of new conditional inducible knockout mouse line for both the TrkA and the p75 receptor, where knockout of either the *NTRK1* or *NGFR* gene respectively is under the control of tamoxifen and restricted to sensory neurons.

Chapter 2: MACS purification as a method to generate pure neuronal cultures from DRG

2.1 Introduction:

As described in the previous chapter (chapter 1.4) there is increasing evidence for the role of a neuroimmune interaction in the processing of acute and chronic pain. It is known that following injury a number of growth factors, inflammatory mediators, chemokines, and cytokines including NGF, bradykinin and prostaglandin E2 (PGE2) to name a few are secreted as components of the "inflammatory soup". However, the mechanism of action of these factors, including NGF, is not fully known e.g. whether the factors bind directly to the peripheral terminals of sensory neurons or there if they act indirectly via non-neuronal cells expressing the ligand receptors of the inflammatory soup. One way to decipher the mechanism of action is purifying specific cell types, such as neurons, for which *in vitro* experiments of individual cell types or co-culture systems can be treated with specific components of the "inflammatory soup", such as NGF.

2.1.1 Current methods of generating pure neuronal and non-neuronal cultures

A number of methods have been described in the literature to purify specific cell types from more complex tissues. These include filtering cells through a BSA or percoll gradient,

immunopanning, fluorescence-associated cell sorting (FACS) and depletion of other cell types using chemical reagents.

A BSA cushion is used in order to remove a variable proportion of satellite glial cells (SGC) from a DRG cell culture preparation. However, this method is only valid to reduce the number of SGCs does not fully purify the neuronal culture, it merely enriches the number of neurons in the culture (de Luca, Faroni, and Reid 2015). Following digestion, dissociation and titration of DRG cells they are filtered and then pipetted carefully onto the top of a 15% BSA cushion and centrifuged to pellet cells to the bottom of the tube through the cushion. Using this method, a proportion of the SGCs within the single cell suspension are retained in the BSA cushion and therefore removed with the supernatant. Passing single cell suspensions through a percoll gradient can also be used to clean up and enrich cultures by removing myelin and large aggregates of cells for a number of tissues (Delree et al. 1989). Delree *et al* for example used a percoll gradient to clean myelin from adult DRG cell suspensions before isolating neurons through a small filter of 10 µm which allows the passage of smaller non-neuronal cells and neuronal cells were retained on the filter itself whereas Nguyen *et al* used a percoll gradient to clean up tissue samples digested from the dorsolateral caudal neocortex (V. Nguyen and McQuillen 2010; Delree et al. 1989).

Performing immunopanning requires a single cell suspension firstly placed onto a negative panning plate coated with antibodies against a cell type to be removed from the suspension for 45 minutes. This step can be repeated with the same or a different negative panning plate before the cell suspension is placed onto a positive panning plate coated with an antibody against the cell type of interest. After incubation for 45 minutes, all unbound cells are removed with the supernatant. Trypsin is added to the positive panning plate and cells are collected and plated as recommended for the cell type of interest (Winzeler and Wang 2013). Zhang *et al* describes how the use of a 2 step immunopanning protocol for retinal ganglionic cells (RGC)

was an effective and robust method for isolating RGCs while maintaining the cells neuronal morphology and expression of well-known marker proteins such as Thy-1. Approximately 70% of RGC attached and survived post plating, and cultures could be maintained for up to 1 month at which point there were only 30% RGC remaining. This 2 step protocol involved 2 rounds of incubation on the negative panning plate coated in goat anti-mouse IgG, on top of which the single cell suspension was placed after incubation with a mouse anti-rat macrophage antiserum to tag all of the macrophages in the culture. Following this the cell suspension was then plated onto the positive panning plate coated in anti-Thy-1 antibody to capture the RGC (X.-M. Zhang et al. 2010).

FACS utilises a panel of antibodies to isolate specific cell types. It has been widely used in the immunology field for many years. Recently, with increasing interest in neuroimmune interactions, it is also increasingly used in the pain field. This includes studies like Zhang *et al* which investigated the recruitment of macrophages in the PNS and the CNS after injury. They took bone marrow transplants from a bone marrow chimeric mouse line which have a GFP tagged cells and injected this into irradiated wildtype C57Bl mice 3 weeks before a dorsal root ganglionectomy was performed. FACS was then used to isolate GFP vs non GFP expressing macrophages to ask whether circulating macrophages were limited to the PNS or if they were also recruited into the CNS as part of a repair mechanism (G. Zhang, Hoffman, and Sheikh 2014). Other studies using FACS have used this method to separate populations of neurons that have been injected with tracers. For example, Reinhold *et al* injected Fluoroemerald (FE) into a nerve proximal to the site of a chronic constriction injury and 1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (Dil) into the plantar skin of the hindpaw in order to isolate and investigate the changes in damaged and intact neurons after injury (Reinhold et al. 2015).

Some methods purifying neurons involve the depletion of non-neuronal cells using chemical reagents. Kar et al collected superior cervical ganglia and despite using MACS technology to dissociate cells, neurons were enriched by treating cultures with 5-fluoro-2'-deoxyuridine, an anti-mitotic inhibitor, to prevent the growth of non-neuronal cells in the culture (Kar et al. 2013). This same technique has also been used by Ross *et al* after treatment of DRG cultures for 3 weeks (Ross et al. 2001). When using fluorodeoxyuridine for neuronal enrichment of DRG cultures three treatments within two weeks were needed and even then the achieved enrichment did not reach the experimental requirements for purity. Therefore, this method has later been improved with a treatment of Ara-C (R. Liu, Lin, and Xu 2013). Using Ara-C it was found that a single high dose treatment of anti-mitotic inhibitors flurorodeoxyuridine and Ara-C for 72 hours was enough to obtain 99% neuronal purity from DRG cultures after only one week in culture. Even though non neuronal cells survived and proliferated in culture post 24 hours, a large scale death of these cells was found with no or minimal toxic effect observed on the survival of neuronal cells (R. Liu, Lin, and Xu 2013).

A different approach to the above methods which involves the isolation of the cell of interest from tissue with subsequent purification is the generation of new cell line models which are able to replicate the behaviour and responses of a particular cell type *in vitro*. Although there are a number of cell lines that have been used in the past to study neurons, including PC12 and SH-SY5Y cell lines, recently there has been work describing a new method for reprogramming fibroblasts to differentiate into neurons. Wainger *et al* describes how a mixture of five transcription factors can be used to re-programme fibroblasts into nociceptors. The advantage of this method being that it is easier to isolate and collect large numbers of fibroblasts which can proliferate in culture compared to neuronal cultures. Fibroblasts treated with this mixture of transcription factors, express protein and ion channels similar to those found in terminally differentiated neurons of adult mice and respond to inflammatory and chemotherapy induced

neuropathy as observed by an increase in the expression of TRPV1 following the treatment of cultures with the inflammatory mediator prostaglandin E2 and oxaliplatin respectively (Wainger et al. 2014).

In addition to reprogramming fibroblasts, new cell lines of sensory neurons have been obtained using the immortimouse model, a transgenic mouse line which can be used to isolate immortal cell lines. The benefit of this technique being large numbers of nociceptors for *in vitro* studies can be obtained without the need for large numbers of animals. Doran *et al,* 2015 collected E12.5 embryos and DRG were isolated with the aim to collect and clone sensory neuron progenitor cells. E12.5 embryos were used as it is known that at E12.5 proprioceptor and low threshold mechanoreceptors are terminally differentiated, whereas the nociceptors are still dividing. This study found that the newly isolated MED17.11 cell line was capable of undergoing neurite outgrowth and correlated best to sensory neuron progenitors as testing showed their cytochemical and pharmacological properties to be consistent with nociceptive lineage neurons. However, these cell types also had markers for mechanoreceptors and proprioceptors suggesting that they may have multimodal potential and could be targeted to a more specific lineage using a targeted differentiation medium for example with specific growth factors for a subtype of nociceptors (Doran et al. 2015).

2.1.2 MACS has been used to purify a number of neuronal and non-neuronal cell types

Recently, Miltenyi produced a commercially available method which allows magnetic assisted cell sorting (MACS) of cell suspensions through the process of positive or negative selection. Originally, this method was developed for use in the immunology field and was first used to separate B cells from peripheral blood samples (Abts et al. 1989). It was developed as a more powerful tool to isolate rare or highly abundant cell types compared to other purification

methods such as FACS which is time consuming and laborious (Miltenyi et al. 1990). Recently however, MACS technology has developed and begun branching out into other fields such as neuroscience.

Currently, in the neuroscience field MACS is being used to isolate a number of cell types including retinal ganglionic cells (RGC), neurons, astrocytes, microglia and macrophages (for neuroimmune interactions) to name a few.

Haraguchi *et al* used MACS purification to isolate microglia, macrophages and neutrophils in order to better understand the neuroimmune interactions, specifically investigating the role of transient receptor potential melastatin 2 (TRPM2), a nonselective calcium permeable cation channel, in neuropathic and inflammatory pain. They incubated plantar skin and lumbar cord tissue samples either with PE-labelled anti-CD11b antibodies for microglia, PE-labelled rat anti-F4/80 antibody for macrophages or biotin-labelled rat anti-Gr-1 for neutrophils. Anti-PE microbeads or anti-biotin microbeads were then added to the respective solutions to isolate either microglia, macrophages or neutrophils from the sample (Haraguchi et al. 2012). A number of other studies have used these antibodies. This includes the isolation of microglia using MACS anti-CD11b antibodies to investigate activation of microglia (J. Lee et al. 2008) and isolation of CD11b/c positive cells from sections of the hippocampus (Williamson et al. 2011). However, in this study the preparation of tissue from the hippocampus in this study involved a two-step use of MACS technology. First anti-myelin microbeads were used to remove myelin from the single cell suspension and the suspension was run through a MACS LD column before the cell suspension from the flow-through was incubated with PE-conjugated mouse anti-rat CD11b/c antibody and anti-PE microbeads. Following this second incubation the cell suspension was then passed through a MACS LS column (Williamson et al. 2011). In addition to this, MACS purification has also been used to isolate retinal ganglion cells (RGCs). It has been shown that incubation of retinal cell suspensions with biotinylated anti-rat Thy-1 and MACS

streptavidin microbeads can effectively enrich RGC from 0.55% to 31% after passing through the MACS column (Shoge et al. 1999). Also, Cizkova *et al* have used MACS technology to generate oligodendrocyte cultures *in vitro*. They used specific A2B5 antibody labelled microbeads to isolate neural stem cells from E16 spinal cord samples, which differentiated in culture. They found that this method was effective in enriching oligodendrocyte cultures from 6-8% before to 58-61% following MACS and contained a mixture of both oligodendrocyte precursors and mature oligodendrocyte cells as observed by specific cell markers (Cizkova et al. 2009).

Vroemen *et al* described the use of MACS to purify p75 positive Schwann cells from peripheral nerve biopsies with the aim to use these Schwann cell cultures in the future to investigate regeneration, axon repair and remyelination. They described two different methods of MACS purification to enrich Schwann cells. First they used anti-p75 monoclonal antibodies, similar to the technique previously described which has been reported to take 3-6 weeks to enrich Schwann cell cultures (Tuszynski et al. 1998). Instead they used here this anti-p75 monoclonal antibody with microbead linked rat anti-mouse IgG1 to tag all p75 positive Schwann cells before passing the cells through the MACS MS+ column within the miniMACS magnet to collect tagged cells and isolate them using positive selection. In the second method anti-Thy-1 IgG1 with microbead linked rat anti-mouse IgG1 antibodies were used to tag fibroblasts in the single cell suspension and deplete them using the MACS MS+ column within the miniMACS magnet. In this way, Schwann cells of interest were collected by negative selection where tagged fibroblasts were retained in the column and Schwann cells passed through the MACS column and were collected in the flow through. The first method resulted in a better enrichment of p75 positive Schwann cells than the second. However, the second method showed weakness when used with adult tissue, but it worked better when tested with neonatal tissue. Overall, compared to previous purification methods this method was significantly faster where 9 days

after collection of peripheral nerve biopsies cultures were purified. (Vroemen and Weidner 2003).

Finally, MACS has been used to purify neural precursors from the ventricular zone of brains from human foetuses. Tissue was homogenised and incubated with PE-conjugated anti-CD133+ antibodies and anti-PE magnetic beads and run through a MACS MS column in the miniMACS separator. FACS and immunocytochemistry was used to assess the enrichment after MACS purification. It was found that the cell suspension post MACS contained 85% neural precursor cells compared to 6-8% directly after dissection, with no change in the viability of the cells (Yu et al. 2004).

Using a non-neuronal antibody cocktail, neurons can be isolated from tissues by negative selection using MACS technology, as used in my work/ the current study. Briefly, this commercially available technique involves the incubation of dissociated and triturated DRG samples with firstly a cocktail of non-neuronal antibodies with biotin tag against astrocytes, oligodendrocytes, microglia, fibroblasts and endothelium cells to tag all non-neuronal cells. An anti-biotin secondary antibody with a microbead tag is then added to the cell suspension which gives cells a magnetic charge. Cell suspensions are then run through a LD column containing ferromagnetic spheres which in combination with a large magnet amplifies the magnetic field within the column. This holds all non-neuronal tagged cells in suspension in the column while untagged neurons flow through the column by negative selection (**Fig 2.1**).

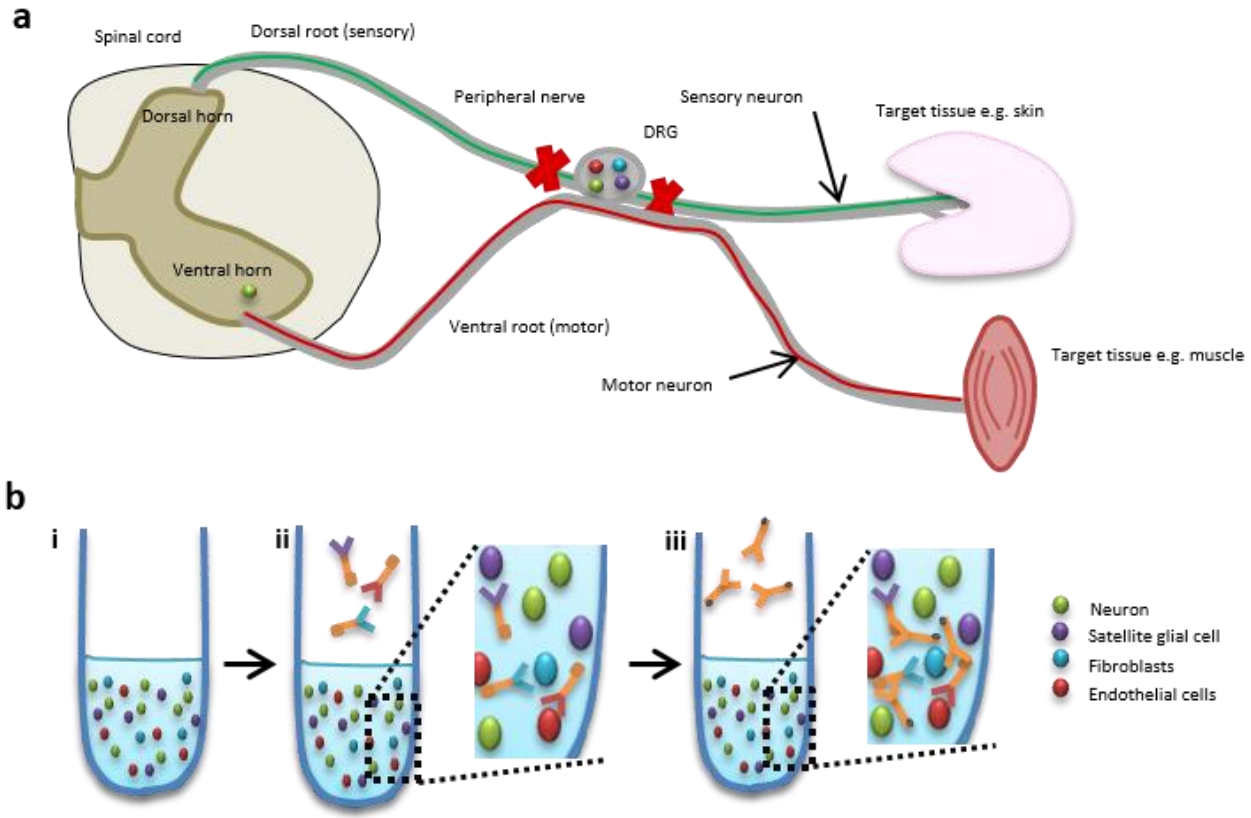

a
Spinal cord
Dorsal root (sensory)
Dorsal horn
Peripheral nerve
Sensory neuron
DRG
Target tissue e.g. skin
Ventral horn
Ventral root (motor)
Target tissue e.g. muscle
Motor neuron
b
i
ii
iii
Neuron
Satellite glial cell
Fibroblasts
Endothelial cells
c

Figure 2.1 Schematic of MACS purification (Miltenyi biotech). ***a-*** *Schematic showing the anatomical location of DRG neurons containing the cell bodies of sensory primary afferent neurons. All DRG from cervical to lumbar regions collected. Red crosses indicate the parts of the sensory nerve fibres cut either side of the DRG.* ***b i-*** *collagenase digestion and trituration to single cell suspension* ***ii-****incubation with cocktail of non-neuronal biotin conjugated primary antibodies. Cocktail contains a mixture of antibodies against astrocytes, oligodendrocytes, microglia, endothelial cells and fibroblasts.* ***iii-****incubation with an Anti-biotin micro beads conjugated secondary antibody* ***c*** *LD columns are held in a strong magnetic bar. Ferromagnetic spheres in LD columns amplify the magnetic field within the column and hold micro bead tagged non-neuronal cells in suspension. Untagged neuronal cells flow through the column and are collected in the eluate through negative selection.*

2.1.3 Aim of the chapter

The overall project aims at deciphering the mechanism of action of NGF in pain. Neurons and non-neuronal cells have been reported to express the NGF receptors TrkA and p75 (Usoskin et al. 2014; Thakur et al. 2014; Aloe et al. 2012; Richner et al. 2014; Ebenezer et al. 2007). It is unknown whether NGF signals peripheral sensitivity following injury via direct binding to neurons or an indirect effect from non-neuronal cells. In order to distinguish these two possibilities, it is essential to have purified neuronal cultures. The aim of this part of the project is therefore, to optimise a method to purify sensory neurons from dorsal root ganglia (DRG) which contain the cell bodies of sensory neurons innervating the periphery. We decided to use MACS technology with the non-neuronal antibody cocktails to isolate neurons by the process of negative selection. Since this protocol had not previously been used to isolate sensory neurons from DRG, we optimise and characterise this protocol here.

2.2 Methods:

2.2.1 Animals

All animals were obtained from Charles river and housed in a designated facility and maintained in accordance with ASPA and Home Office regulations. Animals were kept in a 12hr light dark cycle and fed *ad libitum*. All animals were sacrificed using schedule 1.

2.2.2 DRG cultures

All (or most) dorsal root ganglia were collected from adult C57Bl mice, incubated at 37°C for 45 minutes and dissociated using an enzyme mixture of 3 mg/ml dispase (Roche, 04942078001), 0.1% collagenase (Sigma Aldrich, C9891) and 200 U/ml DNAseI (Roche, 10104159001) in F12 medium (Life Technologies, 21765029). Cells were centrifuged for 15 seconds at 800 rpm and the supernatant discarded. The cell pellet was then gently triturated in 1 ml of F12 medium before brief 15 second centrifugation at 800 rpm. Supernatant was then collected and the titration procedure of pellet resuspension and supernatant collection was repeated 4 times. Supernatant was then filtered through a 70 µm filter and centrifuged for 8 minutes at 1000 rpm. Supernatant was discarded and cell pellet re-suspended in 200µl of plating medium prior to counting and plating. DRG cultures were plated at a density of approximately 3000 neurons in a 70 – 80 µl volume per well of 4 well plates containing 13mm diameter coverslips. Coverslips/ plates were pre-coated with 1:10 poly-L-lysine (PLL) (Sigma Aldrich, P4707) in dH_2O overnight at 4°C and 1:10 growth factor reduced (GFR) matrigel (BD bioscience, 356230) in F12 for 2 hrs at room temperature (RT). Plates were left with the added cells at room temperature for 5 min to allow cells to settle before being placed in the incubator (37^oC 5% CO2) for 1 hour to allow cells to attach prior to addition of 400 µl of plating medium. For DRG cultures without purification the plating medium used was BS medium (10% FBS (Sigma Aldrich, F9665), 1% N2

supplement (Life technologies, 17502-048), 1x PenStrep (Sigma Aldrich, P4333) in Ham's F12 with L-Glut (Life technologies, 21765029)). Cells were fixed 24 hours after plating.

2.2.3 Magnetic-assisted cell sorting (MACS)

Following dissociation and titration (as described above) cells were centrifuged for 8 minutes at 1000 rpm and supernatant discarded. The cell pellet was re-suspended in 2 ml of DPBS (Gibco, 14190144) and centrifuged at 1000 rpm for 7 minutes. Supernatant was discarded and cells were re-suspended in 130 µl of 0.5% w/v BSA in MACS buffer (wash buffer used throughout protocol) and 30 µl of the non-neuronal biotin antibody cocktail (commercially available) against astrocytes, oligodendrocytes, microglia, fibroblasts and endothelium was added and incubated at 4°C for 5 minutes (Miltenyi biotec, 130-098-752). Cell suspension was topped up to 2 ml final volume with MACS buffer and centrifuged at 1000 rpm for 7 minutes. Supernatant was discarded, and cells re-suspended in 130 µl of MACS buffer and 30 µl of anti-biotin microbeads (Miltenyi biotec, 130-098-752). Cells were incubated at 4°C for 10 minutes before topping up to 500 µl with MACS buffer. For neuronal separation this protocol utilises LD columns from Miltenyi Biotech to purify neurons by negative selection, see **Fig 2.1** for schematic. LD columns were primed with 2 ml MACS buffer in the QuadroMACS separator before 500 µl of the cell suspension was added to the column. Meanwhile, 1 ml of MACS buffer was added to the tube previously containing the cell suspension to wash out any remaining cells and added to the column once the cell suspension had run into the column, followed by a washing through with 1.5 ml MACS buffer added directly to the column. Eluate was centrifuged at 1000 rpm for 8 minutes and cell pellet re-suspended in plating medium. The content of the plating medium varied depending on the experiment: Ham's F12 with L-Glut, BS (described above) or B27 supplemented (2% B27 supplement (Life technologies, 17504-044) and 1xPenStrep in F12 medium). Approximately 3000 cells per well were seeded and left at room temperature for 5 min before being placed in the incubator (37^{o}C 5% CO2) for 1 hour

prior to addition of 400 µl of plating medium. For experiments involving NGF treatment, control conditions were plated in B27 medium and the experimental conditions contained different dilution of 100 µg/ml NGF in 0.1% BSA in HBSS (Cambridge Bioscience, GFM11-1000) diluted in B27. Cultures were left in the incubator for 24 – 48 hours. Cultures that were left more than 24 hours, media was changed after 24 hours.

2.2.4 Percol purification

DRGs were dissociated, titrated, filtered and centrifuged for 8 mins at 1000 rpm as mentioned previously. While cells were centrifuging, the percoll gradient is prepared. In duplicate, prepare 28% percol (GE Healthcare, 17-0891-01) in F12 medium (no additives) and add 4ml to each tube. In a separate tube prepare 12.5% percol in HBSS (Gibco, 14025-050) and gently layer 4ml over the 28% percol layer already in your prepared tubes. Once the cell suspension has been centrifuged, remove the supernatant and re-suspend the cell pellet in 1 ml of HBSS. Carefully add 0.5 ml of cell suspension over the percoll gradient and centrifuge for 10 min at 1300 rpm, slow the centrifuge using the soft break for this step. Cells will pass through the gradient to the bottom of the tube and the debris and myelin will collect at the percoll interface. Discard the top 4.5 ml including the debris and add 4ml of F12 medium to the remaining solution in the tube. Centrifuge for 6 min at 1000 rpm. Remove the supernatant and re-suspend in BS plating medium and plated onto glass coverslips coated in PLL and laminin. Coverslips/ plates were pre-coated with 1:10 PLL (Sigma Aldrich, P4707) in dH_2O overnight at 4°C and 1:100 laminin (Sigma, L2020) in F12 for 2 hrs at 37^{o}C. Plates were left with the added cells at room temperature for 5 min to allow cells to settle before being placed in the incubator (37^{o}C 5% CO2) for 1 hour to allow cells to attach prior to addition of 400 µl of plating medium.

2.2.5 Immunocytochemistry

Equal volume of warm (37^{o}C) 4% PFA was added to culture medium (final concentration 2% PFA) on PLL and matrigel coated coverslips (described above) and cells were incubated for 20min at 37^{o}C. Coverslips were then washed twice with 1xPBS and stored at 4^{o}C in 1xPBS prior to immunostaining. Coverslips were blocked with 10% normal donkey serum (NDS) (Abcam, ab1666643) in PBS-Triton X-100 (0.2% triton-100 and 0.1% sodium azide in 1xPBS) for 1 hour at RT. Blocking solution was then removed and replaced with primary antibody solution (antibody in 0.2% PBS-Triton X-100; please see antibody dilutions below) and left for 1 hour at RT. Cells were then washed 3 x 5min with 1xPBS before secondary antibodies diluted 1:1000 in 0.2% PBS-Triton X-100 were added and incubated for 1 hour at RT in the dark before washing 3x 5min with 1xPBS. Coverslips were then mounted onto microscope slides using hard set mounting media with DAPI (vector laboratories, H-1500).

Primary antibodies were diluted as follows: 1:1000 anti-β-III tubulin mAb (Promega, G712A) for all experiments except for 1:1000 anti-β-III tubulin rabbit polyclonal (Abcam, ab18207) used as a counterstain for Isolectin B4, 1:1000 anti-glutamine synthetase (GS) Rb pAb (Abcam, ab49873) and 1:500 Isolectin B4 biotin conjugate (Sigma Aldrich, L2140).

The following secondary antibodies were used: donkey anti-mouse IgG (H+L) Alexa Fluor® 488 (ThermoFisher, A21202) and donkey anti-rabbit IgG (H+L) Alexa Fluor® 546 (ThermoFisher, A10040). In experiments with percoll purification, MACS vs flow-through and plunger and β-III counterstain for IB4 donkey anti-rabbit IgG (H+L) Alexa Fluor® 568 (ThermoFisher, A10042) and streptavidin, Alexa Fluor® 488 conjugate (ThermoFisher, S32354) was used.

2.2.6 Viability assay

Viability assays of DRG cultures were carried out using Live/dead ® reduced biohazard viability/cytotoxicity kit #1 (Life technologies, L-7013). Staining was performed according to the

protocol for staining adherent cells on coverslips. Coverslips were mounted using hard set mounting media with DAPI (vector laboratories, H-1500). This kit uses a highly membrane permeant SYTO® 10 green fluorescent nucleic acid stain which labels all cells and membrane impermeant DEAD Red™ red fluorescent stain which only labels cells with a compromised cell membrane.

2.2.7 Imaging and analysis:

Mosaic images were acquired at 200x using Carl Zeiss microscope and Axiovision software. Percentages of cell proportions were measured in Fiji using the cell counter plugin across a minimum of three fields for each individual experiment. Viability was also measured using the Fiji cell counter plugin where a minimum of 50 neurons (based on the size and shape of the cell) were counted across a minimum of two fields for each experimental replicate. Finally, cell size distribution was measured by circling the perimeter of neurons and calculating the Feret diameter from the soma area.

Neurite outgrowth assay was optimised and 40 neurons (20 per technical replicate) per experimental replicate were traced using Fiji. All individual neurites were traced along each neurite projecting from the soma to their longest branch point and data used to measure the longest and shortest, the mean length, number of neurites and the total neurite outgrowth from each of the longest branches. Following this, all remaining branches were traced and from this the overall neurite outgrowth was calculated and the overall neurite outgrowth normalised to the number of neurites (**see Fig 2.6a**).

2.2.8 Statistics

Graphs were plotted and statistical tests performed using GraphPad Prism 7 software. One and two-way ANOVA were performed to analyse cell size distribution and neurite outgrowth measurements with data corrected for multiple comparisons with Tukey or Bonferroni post-

hoc test where appropriate. Finally, linear regression analysis was performed to compare neurite outgrowth parameters.

2.3 Results

2.3.1 Purification of DRG cultures using a percoll gradient does not remove satellite glial cells

Passing dissociated DRG neurons through a percoll gradient is a commonly used method to clean up and purify DRG cultures. Therefore, we first tested percoll gradients as a method to purify DRG neurons. Following dissociation and titration, DRG cell suspensions were passed through a percoll gradient and then cultured for 24 hours *in vitro*. Percoll purified cultures although being free of debris and myelin were found to be 20.1% (±2.1%) neuronal, 71.1% (±3.2%) satellite glial cells (SGC) and 8.7% (±1.4%) other nuclei (**Fig 2.2a and b**). Measuring the cell size distribution showed a loss in the medium and large diameter neuronal population compared to the small diameter neurons where small diameter neurons are defined as <30um, medium diameter 30-40um and large diameter neurons are >40um. Small diameter neurons vs medium diameter neurons p=0.0029 and small diameter neurons vs large diameter neurons p=0.0014. Two-way ANOVA with Tukey post-hoc (**Fig 2.2c**).

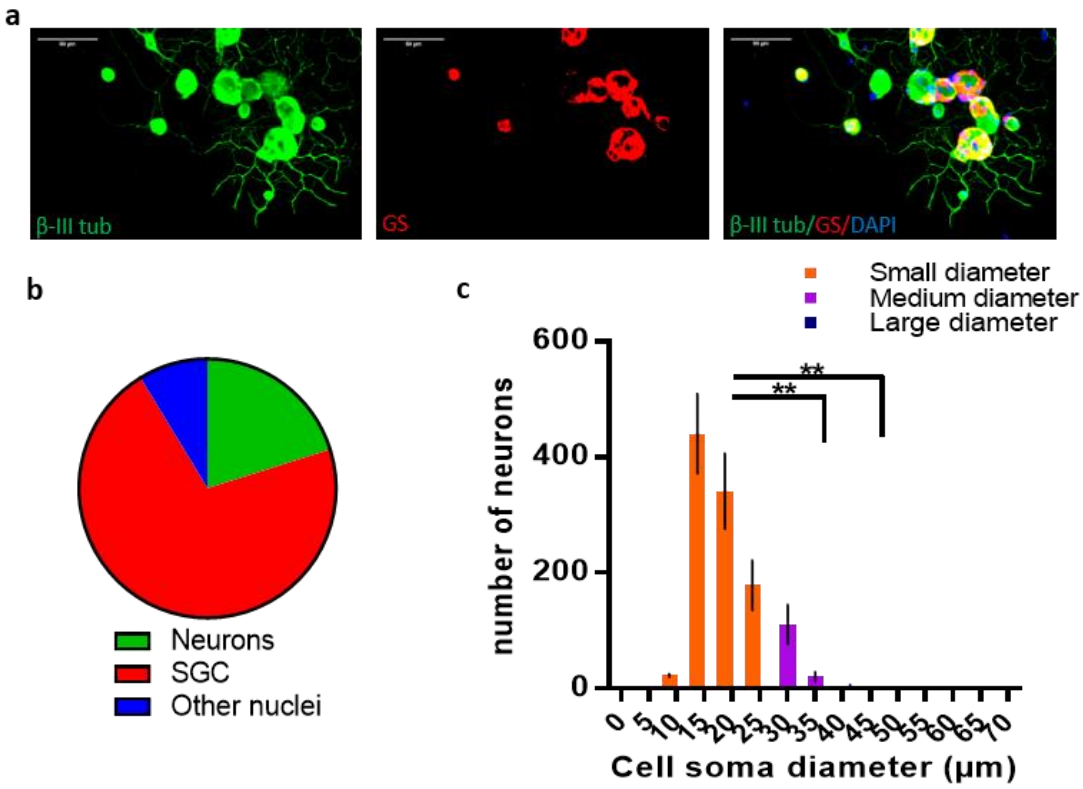


Figure 2.2 Passing DRG neurons through a percoll gradient prior to plating is not sufficient to purify DRG neurons in culture*. **a-** representative immunocytochemistry (ICC) images of dissociated DRG passed through a percoll gradient. Staining with β-III tubulin for neuronal cells, glutamine synthatase (GS) for SGC cells and DAPI for nuclei. **b-** quantification of percentage of neurons (20.1% ± 2.1%) and SGC (71.1% ± 3.2%) in percoll purified cultures, n=3 independent cultures. **c-** cell size distribution of neurons. One-way ANOVA with Tukey post hoc test. Small diameter vs medium diameter p=0.0029, small diameter vs large diameter p=0.0014.*

2.3.2 Optimisation of Magnetic-Activated Cell Sorting

Magnetic-Activated Cell Sorting (MACS) has been increasingly used in the neuroscience field to isolate specific cell populations. We decided to use this technology to isolate sensory neurons from DRG. As described previously, this involves the incubation of dissociated and triturated

DRG samples with firstly a commercially available kit with a cocktail of non-neuronal antibodies with biotin tag against astrocytes, oligodendrocytes, microglia, fibroblasts and endothelium cells to tag all non-neuronal cells and then an anti-biotin secondary antibody with a microbead tag which gives cells a magnetic charge (**Fig 2.1**). Please note that as a commercially available kit there are no details on the antibodies used to target specific populations in this kit. In addition there is no mention from the company about this kit targeting satellite glial cells however, there is evidence from this study that these cells are also targeted. Neurons are then collected by negative selection after the cell suspension is run through an LD column held in a magnetic field. Standard DRG cultures are normally grown on poly-L-lysine(PLL) coated coverslips secondarily coated in laminin at a concentration of 1:100 in F12 medium for 2 hours at 37^{o}C. Cells grown on laminin were fixed with 4% PFA for 10 minutes at room temperature. However, growing MACS purified neurons on laminin and fixing in 4% PFA was not optimal for MACS purified cultures as MACS purified neurons did not adhere well to glass coverslips coated with PLL and laminin. In order to address this a number of coating substrates were tested including ConcanavalinA, collagen and martigel (**Table 2.1**) A thin layer of 1:10 matrigel incubated for at least 1 hour at RT on PLL coated coverslips was found to be the optimal substrate for purified neurons to be adherent in culture, however, during the fixing of cells with 4% PFA for 10 min at room temperature a loss of cells was observed. Matrigel is a temperature sensitive regent that is liquid between 4^{o}C and 21^{o}C and solidifies below 4^{o}C and above 21^{o}C. We hypothesised that fixation of the cells at RT could have lowered the temperature of the culture allowing the matrigel to liquefy prior to the cells becoming adequately fixed. In addition, high concentrations of PFA have been found to destabilise the matrigel matrix. Therefore, the fixing protocol was altered until an optimal fixation of warm 2% PFA for 20 minutes at 37^{o}C was found (**Table 2.1**).

Coverslip coating	Fixing	Comments
1:100 laminin	4% PFA	Neurons do not adhere or sprout in culture
Concanavalin A in 1:100 laminin	4% PFA	Neurons adhere and sprout in culture but washed off during fixing.
Matrigel – thin gel neat	4% PFA	Neurons adhere but washed off during fixing.
Matrigel – thin coating 1:25	4% PFA	Neurons adhere but washed off during fixing.
Matigel – thin coating 1:10	4% PFA	Neurons adhere but washed off during fixing.
Matigel – thin coating 1:10	2% Glutaraldehyde	Neurons remaining following fixing but high levels of background staining during ICC
Matigel – thin coating 1:10	2% PFA (warm) + 1% Glutaraldehyde	Neurons remaining following fixing but high levels of background staining during ICC, less that 2% Glutaraldehyde alone.
Matigel – thin coating 1:10	2% PFA (warm)	Neurons remaining following fixing, much reduced levels of background staining compared to addition of Glutaraldehyde.

Table 2.1 – Testing coverslip substrates and fixing methods for MACS purified DRG neurons in vitro.

2.3.3 MACS LD column alone is not sufficient to purify neurons and has no negative effect on cell survival

In order to determine what effect the MACS LD column alone has on the purity and the viability of neurons, DRGs were taken and dissociated as described in the methods, then the MACS protocol was carried out in the absence of the MACS antibodies. In substitution of the non-neuronal cocktail and the biotin conjugated antibodies, cells were resuspended in 150µl of MACS buffer as opposed to 130µl and then run through the MACS LD column and referred to here as "mock MACS". Cells were fixed after 2 days in culture and the number of neurons counted at this point. Mock MACS cultures were found to be 8.3% (±4.8%) neuronal, 54% (±9.2%) SGC and 37.7% (±5.9%) containing other cells (other nuclei) (**Fig 2.3a and c**). The neurons in these culture were found to be 98.2% (±0.6%) viable using a live dead viability kit (**Fig 2.3b and e**). Measurement of cell size distribution showed an approximately normal distribution of small, medium and large diameter neurons with a trend towards a reduction in the number of large diameter neurons compared to a standard DRG cultures. There was no significant difference between groups as measured by one-way ANOVA with Bonferroni's post-hoc test (**Fig 2.3d**). Taken together, this suggests that the MACS LD column alone is not sufficient to purify neuronal cultures, however, it also does not cause any cell death.

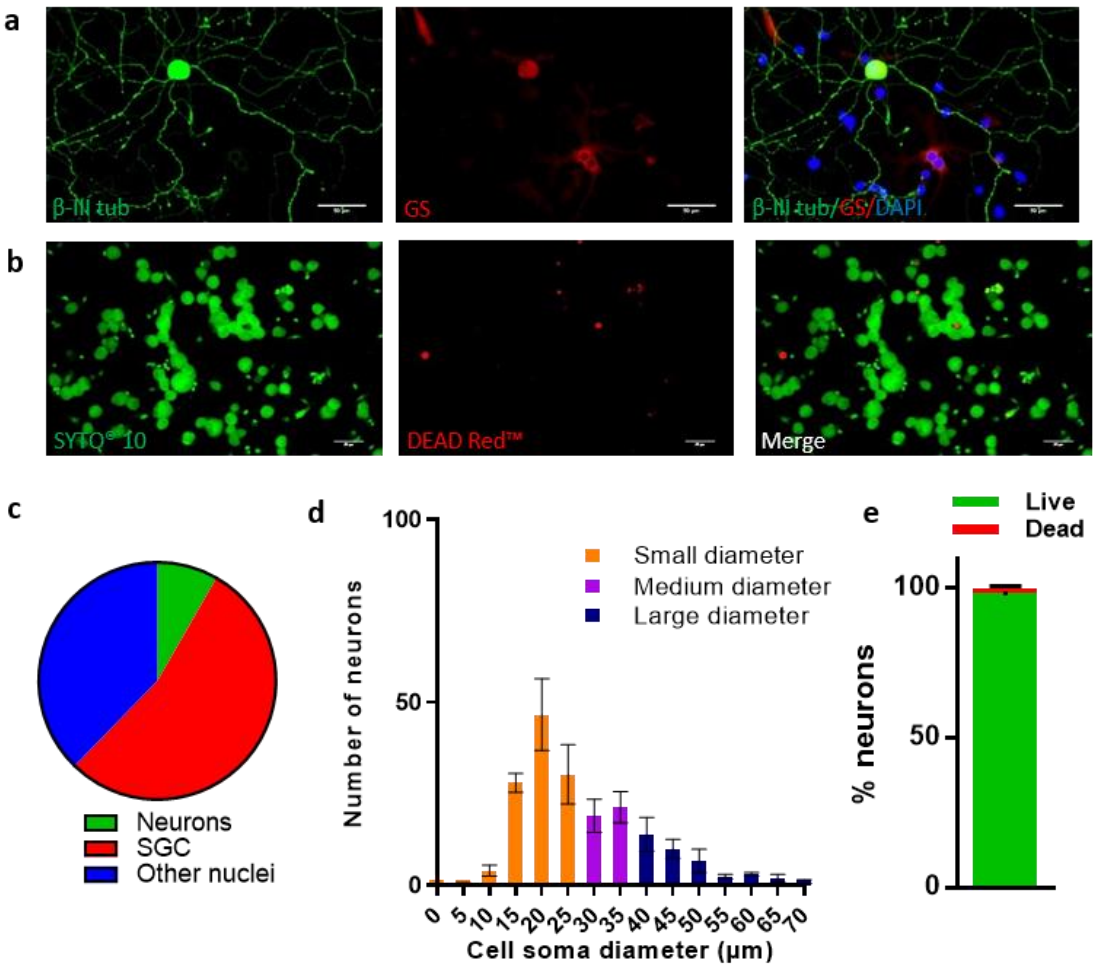


Figure 2.3 The LD column alone is not sufficient to purify neuronal cells and does not induce cell death. a- *representative ICC of DRG neurons purified through a mock MACS through the LD column in the absence of non-neuronal biotin conjugated primary and anti-biotin micro bead conjugated secondary antibodies. Stained with β-III tubulin for neurons and GS for SGC.* ***b-*** *representative ICC of mock MACS purified DRG stained with viability dyes SYTO® 10 live dye and DEAD Red™.* ***c-*** *Quantification of percentage of neuronal cells (8.3% ± 4.8%) and SGC (54% ± 9.2%) following mock MACS purification.* ***d-*** *cell size distribution for mock MACS.* ***e-*** *quantification of viability assay 48 hours in culture, 98.2% live ± 0.6%. All n=3 independent cultures.*

2.3.4 MACS purification enriches viable neuronal cultures

To see if MACS purification can effectively purify sensory neuron cultures from DRGs we first compared the percentage of neurons in both non-MACS and MACS purified cultures left for 24 hours in BS medium. DRG cultures without purification were found to be 11.9% (±0.5%) neuronal, 38.1% (±0.5%) SGC and 50% (±0%) other cells. MACS purified cultures however were 90.1% (±1.4%) neuronal, 0.5% (±0.8%) SGC and 9.4% (±0.8%) other cells (**Fig 2.4a, d and f**). Measurement of cell viability using a live dead kit showed no significant difference in viability of MACS purified neurons compared to non-MACS purified neurons, one-way ANOVA with Tukey post-hoc test. After 48 hours in culture, DRG cultures without MACS purification were seen to be 92.6% (±3.2%) viable cells compared to an average 93.5% (±2.7%) viability for MACS purified cultures, cultured for short-term (1 or 2 hours) or long-term (24, 48 or 72 hours) period of time (**Fig 2.4b and c**).

2.3.5 MACS purified cultures contain mainly small diameter neurons

When performing MACS purification, from my own observation, the cell yield was greatly reduced compared to the yield from a DRG culture not purified. To investigate if the cell loss was widespread or limited to one specific population we measured the cell size distribution of neurons after 24 hours in culture. Compared to non-MACS DRG cultures (**Fig 2.4e**) we observed that MACS purified neurons showed a shift towards small diameter neurons, the result of a significant loss in medium and large diameter neurons (**Fig 2.4g**). Small diameter neurons vs medium diameter neurons p=0.006 and small diameter neurons vs large diameter neurons p=0.004, one-way ANOVA with Bonferroni's post-hoc (**Fig 2.4fg**). To assess whether the MACS column is selective for one subset of small diameter neurons MACS purified neurons, 24 hours in culture, were stained for Isolectin B4 (IB4)– a marker for non-peptidergic

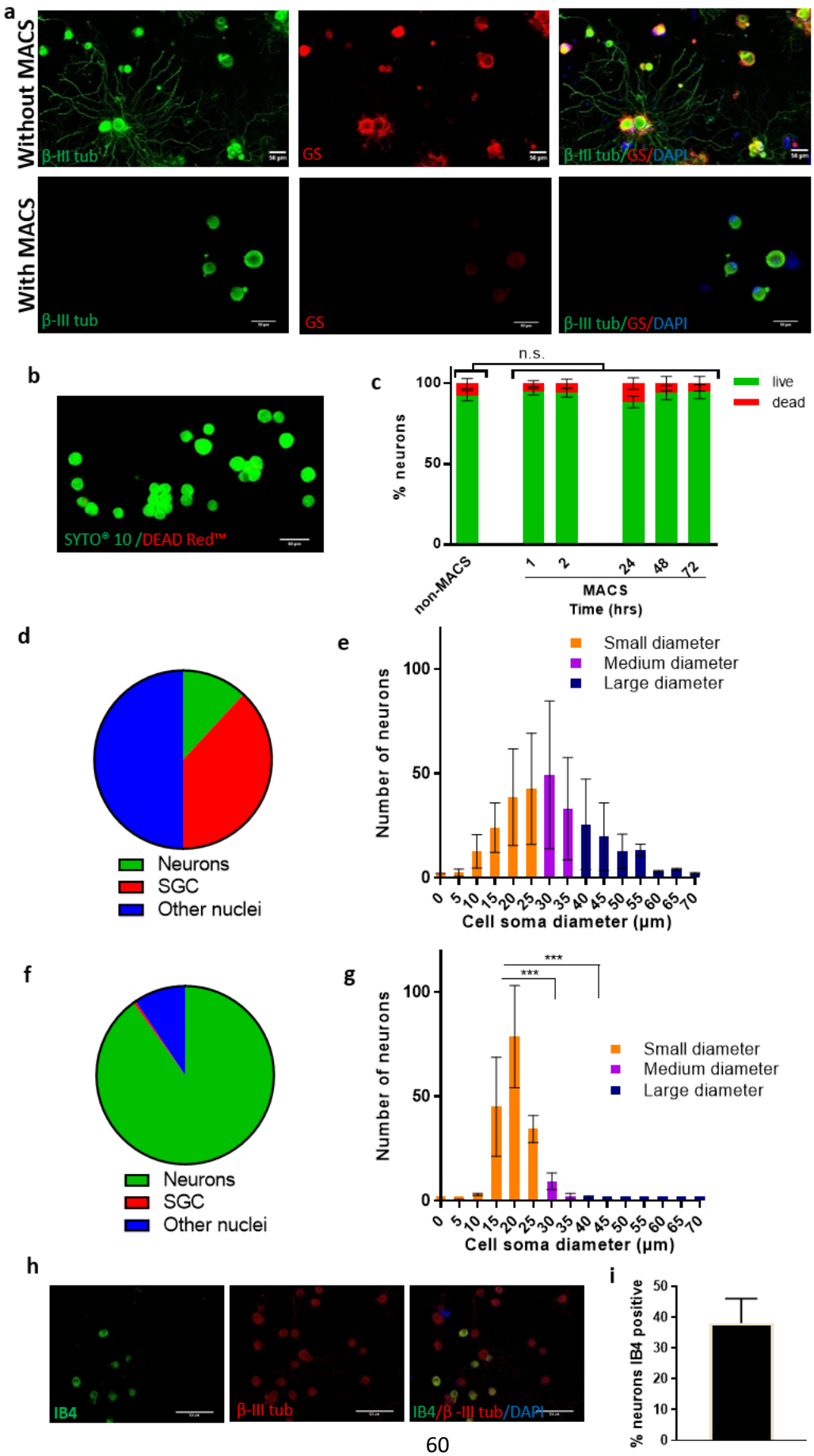
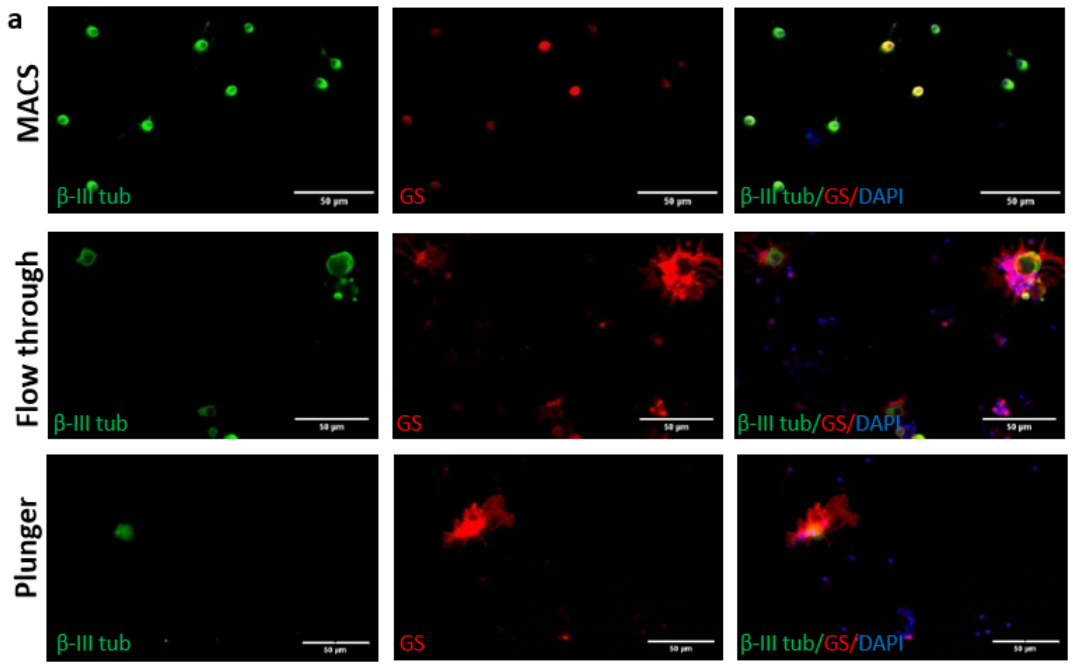

a
Without MACS
β-III tub
GS
β-III tub/GS/DAPI
With MACS
β-III tub
GS
β-III tub/GS/DAPI
b
SYTO® 10 /DEAD Red™
c
n.s.
live
dead
% neurons
100
50
0
non-MACS
1
2
24
48
72
MACS
Time (hrs)
d
Neurons
SGC
Other nuclei
e
Small diameter
Medium diameter
Large diameter
Number of neurons
100
50
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Cell soma diameter (μm)
f
Neurons
SGC
Other nuclei
g
***
***
Small diameter
Medium diameter
Large diameter
Number of neurons
100
50
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Cell soma diameter (μm)
h
IB4
β-III tub
IB4/β -III tub/DAPI
i
% neurons IB4 positive
50
40
30
20
10
0

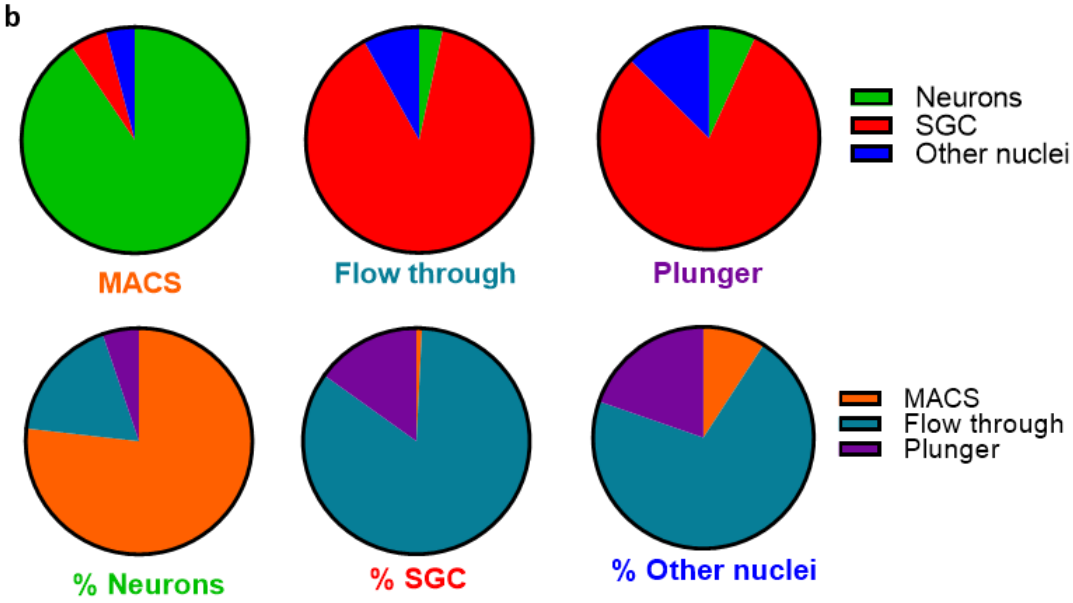
Figure 2.4 MACS purification results in pure neuronal cultures which are viable and consist mainly of small diameter neurons. ***a-*** *representative immunocytochemistry (ICC) of DRG cultures with and without MACS (MACS and non-MACS) purification, stained with β-III tubulin for neurons and GS for SGC 24 hours in vitro.* ***b-*** *Representative ICC for cell viability using SYTO® 10 live dye and DEAD Red™ 3 days following MACS purification.* ***c-*** *quantification of neuron viability for MACS purification at 1, 2, 24, 48 and 72 hours post MACS (average 93.5% live ± 2.7%) compared to non-MACS purification 48 hours in culture (92.6% live ± 3.2%), n=2-4 independent cultures. One-way ANOVA with Tukey multiple comparison.* ***d and f-*** *quantification of representative percentage of neurons and SGC following* ***d*** *non-MACS purification (neurons 11.9% ± 0.5%, SGC 38.1% ± 0.5%) or* ***f*** *MACS purification (neurons 90.1% ± 1.4%, SGC 0.5% ± 0.8%) n=3 independent cultures. Cell size distribution for* ***e*** *non-MACS and* ***g*** *MACS purified DRG neurons show MACS purified cultures to contain mainly small diameter neurons, n=3 independent cultures. One-way ANOVA, Bonferroni's post hoc for MACS cultures small diameter vs medium diameter p=0.006 and small diameter vs large diameter p=0.004.* ***h-*** *Representative ICC for MACS purified neurons stained for IB4 marker for non-peptidergic c-fibre neurons and pan-neuronal marker β-III tubulin 24 hours post MACS purification.* ***i-*** *Quantification of IB4 positive neurons (38.2% ± 7.9%), n=3 independent experiments.*

neurons. MACS purified neurons were found to be 38.2% positive for IB4 (± 7.9%) suggesting that there is no small diameter neuron population bias (**Fig 2.4 h and i**).

MACS LD columns have a pore size of 40µm therefore we hypothesised that the large and medium diameter neurons could be retained in the LD column. We investigated this by collecting three different fractions from the MACS LD column. This included MACS purified neurons, "flow through" from the column collected by releasing the LD column from the magnetic field and adding a wash of 3ml of MACS buffer to the column and finally a "plunger"

fraction where a syringe plunger was used to force through any remaining cells. Each of the fractions were cultured for 24 hours. Firstly, we counted the percentage of neurons that were being collected in each of the fractions. The MACS fraction was found to be 90.6% (±3.2%) neuronal, 5.3% (±1.3%) SGC and 4.1% (±1.9%) other cells compared to the flow through fraction 3.4% (±0.7%) neuronal, 88.5% (±3.5%) SGC and 8.1% (±4.2%) other cells and the plunger fraction 6.9% (±4.1%) neuronal, 80.6% (±5.0%) SGC and 12.6% (±0.9%) other cells. If this is split into cell types we found that neurons represented 76.8% (±1.0%) of the MACS fraction, 18.0% (±1.8%) flow through and 5.2% (±2.8%) plunger. SGC represented 0.8% (±0.4%) of the MACS fraction, 84.1% (±11.4%) flow through and 15.1% (±11.7%) plunger. Other cells represented 9.2% (±10.1%) of the MACS fraction, 71.1% (±9.4%) flow through and 19.7% (±12.5%) plunger fractions (**Fig 2.5a and b**). Cell size distribution for each of these fractions showed that as with MACS purification there is a shift towards small diameter neurons in the flow through and the plunger fractions, and corresponding with the percentages of neurons found in each of the fraction a significant decrease in the number of neurons of certain size. 10µm MACS vs flow through and plunger $p<0.0001$, 15µm MACS vs flow through and plunger $p<0.0001$ and 15µm flow through vs plunger $p=0.0256$, two-way ANOVA with Tukey post-hoc (**Fig 2.5c**).


a
MACS
Flow through
Plunger
β-III tub
GS
β-III tub/GS/DAPI
50 μm


b
MACS
Flow through
Plunger
Neurons
SGC
Other nuclei
% Neurons
% SGC
% Other nuclei
MACS
Flow through
Plunger

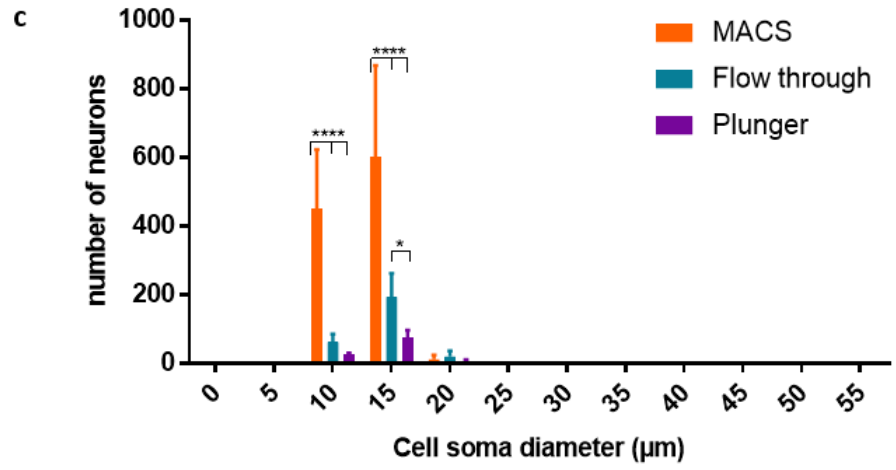

c
number of neurons
1000
800
600
400
200
0
****
****
*
0
5
10
15
20
25
30
35
40
45
50
55
Cell soma diameter (μm)
MACS
Flow through
Plunger

Figure 2.5 A small percentage of neurons are retained in the MACS LD column during purification. a- *Representative immunocytochemistry of neurons and SGC collected within the MACS, flow through and plunger released fractions from the MACS column, cells stained with β-III tubulin pan-neuronal marker and GS for SGC.* ***b-*** *Quantification for representative population of neurons and SGC in the MACS cultures (90.6% ± 3.2% neurons, 5.3% ± 1.3% SGC), flow through (3.4% ± 0.7% neurons, 88.5% ± 3.5% SGC) and plunger (6.9% ± 4.1% neurons, 80.6% ± 5.0% SGC) fractions from the MACS LD column.* ***c-*** *Cell size distribution for MACS, flow through and plunger fractions. Two-way ANOVA, Tukey post hoc **** p<0.0001, * p= 0.0256. All n=3 independent cultures.*

2.3.6 MACS purified neurons exhibit a delay in neurite outgrowth

Since we established that MACS cultures were viable the next step was to determine whether the neurons showed similar neurite outgrowth compared to non-MACS DRG neurons. We developed a neurite outgrowth assay and investigated neurite outgrowth 24 and 48 hours post-MACS purification. The neurite outgrowth assay involved firstly the tracing of all neurites from a cell body to the furthest point, this gives longest, shortest and mean neurite lengths, number of neurites and the total neurite length of the longest neurite branches from each neurite from the soma. Secondarily, the remaining branches of the neuron are traced to estimate the overall neurite outgrowth and the overall neurite outgrowth normalised to the number of neurites (**Fig 2.6a**). We compared each of these neurite outgrowth parameters to the other measurements for all neurons analysed in this chapter and found that there was a correlation between the parameters, some of which were found to be significant. For example, mean neurite length vs longest neurite (R^2=0.974), total neurite length from the longest branch vs total neurite outgrowth (R^2=0.9578) and longest neurite vs overall total normalised to number of neurites (R^2=0.9082). Taken together, conditions which show

significance across multiple neurite outgrowth parameters, particularly highly correlated measurements, in various experiments adds weight to observations as being more biologically relevant (**Supplementary Fig 2.1 and Supplementary Table 2.1**).

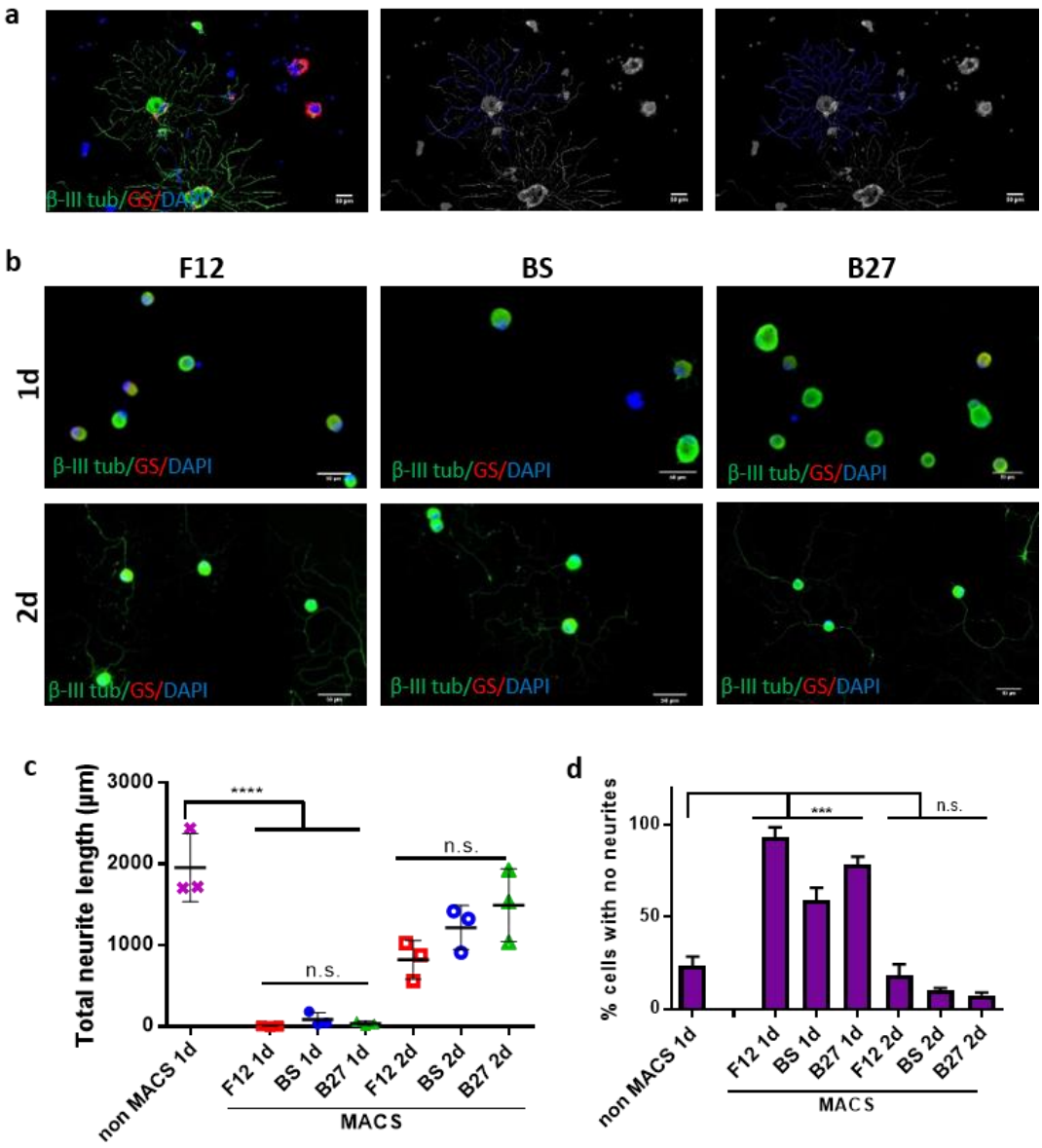


Figure 2.6 MACS purified neurons exhibit a delayed onset for neurite outgrowth compared to standard DRG cultures. a- *representative immunocytochemistry of non-MACS DRG culture left for 1 day in BS medium stained with β-III tubulin pan-neuronal marker and GS (left) and example traces for neurite outgrowth measurements tracing first all neurites to furthest point (middle) and then all remaining branches (right) using Fiji (ImageJ).* ***b-*** *representative ICC for*

MACS purified DRG neurons plated in F12, BS or B27 supplemented medium for 1 or 2 days. ***c-*** *Total neurite outgrowth quantification for MACS purified neurons compared to no MACS purification for different plating mediums. One-way ANOVA, Tukey post hoc, p<0.0001.* ***d-*** *quantification of the percentage of cells with no neurites 1 and 2 days post MACS purification and plating in different culture mediums. Two-way ANOVA, Tukey post hoc p≤0.002. All n=3 independent cultures.*

From personal observation, we observed a significant reduction in the total neurite outgrowth in MACS purified neurons compared to non-MACS purified neurons after 24 hours in culture. We investigated the neurite outgrowth of MACS purified neurons further by culturing purified neurons in three different growth mediums and measuring neurite outgrowth after 24 and 48 hours post-MACS. Compared to non-MACS purified neurons cultured in standard BS medium (1958 ± 420 µm), there was a significant reduction in the neurite outgrowth of MACS purified neurons with F12 (4 ± 4 µm), BS (87 ± 84 µm) and B27 (39 ± 25 µm) medium (average taken from all neurons measured per condition). Non-MACS purified BS vs MACS purified F12, BS and B27 all p<0.0001, one-way ANOVA with Tukey post-hoc (**Fig 2.6a, b and c**). Furthermore, we found that a large percentage of neurons 24 hours post-MACS purification don't show any neurite outgrowth (F12 93% ± 9%, BS 59% ± 12% and B27 78% ± 8%) compared to the non-MACS purified neurons cultured for 24 hours (23% ± 9%) p≤0.002, two-way ANOVA with Tukey post-hoc (**Fig 2.6d**).

Also we found a significant increase in neurite outgrowth 48 hours post -MACS purification (F12 823 ± 238 µm, BS 1220 ± 272 µm and B27 1495 ± 446 µm) compared to 24 hours post-MACS purification, which correlates with a decrease in the percentage of neurons with no neurites (F12 18% ± 10%, BS 10% ± 3% and B27 8% ± 3%). However, there was no significant difference in either the neurite outgrowth or the percentage of neurons with no neurites for

F12, BS or B27 mediums 24 or 48 hours post-MACS purification (**Fig 2.6c and d**). This observation is supported by additional neurite outgrowth measurements of the longest, shortest and mean neurite length, number of neurons, total neurite outgrowth from the longest branch at overall neurite outgrowth normalised to number of neurites with only minor exceptions. There is a significant difference in the length of the longest neurite between MACS purified neurons grown in F12 and B27 mediums after 48 hours in culture ($p=0.0028$), the total length from longest branch ($p=0.014$) and total neurite outgrowth ($p=0.049$). Interestingly, there is also a significant difference in the number of neurites in MACS purified neurons grown in F12 and BS medium for 24 hours ($p=0.0139$) (**supplementary Fig 2.2**).

The observed delay in neurite outgrowth after 24 hours in culture between MACS and non-MACS purified neurons (**Fig 2.6c**) made us question if this was caused by the absence of non-neuronal cells and secreted factors or whether the cells were stressed and in a sort of "recovery" period. To test this conditioned medium (CM) was taken from a non-MACS culture at 24 hours and used to culture MACS purified cultures which were analysed for neurite outgrowth at 24 and 48 hours. First, to ensure that CM does not affect the purity of MACS purified cultures, the percentage of neurons and SGC were counted. MACS purified cells grown in CM were 91.8% (5.6%) neuronal, 0.2% (0.3%) SGC, 8% (5.3%) other cells (**Fig 2.7a and b**). Compared to MACS purified neurons grown for 24 hours in BS control medium (51 ± 69 µm) there was a significant increase in the overall neurite outgrowth in MACS purified cultures grown in CM (779 ± 74 µm) for 24 hours $p=0.0082$, one-way ANOVA with Tukey post-hoc. However, there was no significant difference in the neurite outgrowth between these conditions after 48 hours in culture post-MACS, (BS control 1308 ± 231 µm and CM 1803 ± 301 µm), (**Fig 2.7a and c**). This trend holds for most of the additional neurite outgrowth measurements except for longest ($p=0.0240$), shortest ($p=0.0216$), mean ($p<0.0001$) and

overall neurite outgrowth normalised to number of neurites (p=0.0002) which show a significant increase between BS and CM after 48 hours in culture (**Supplementary Fig 2.3**).

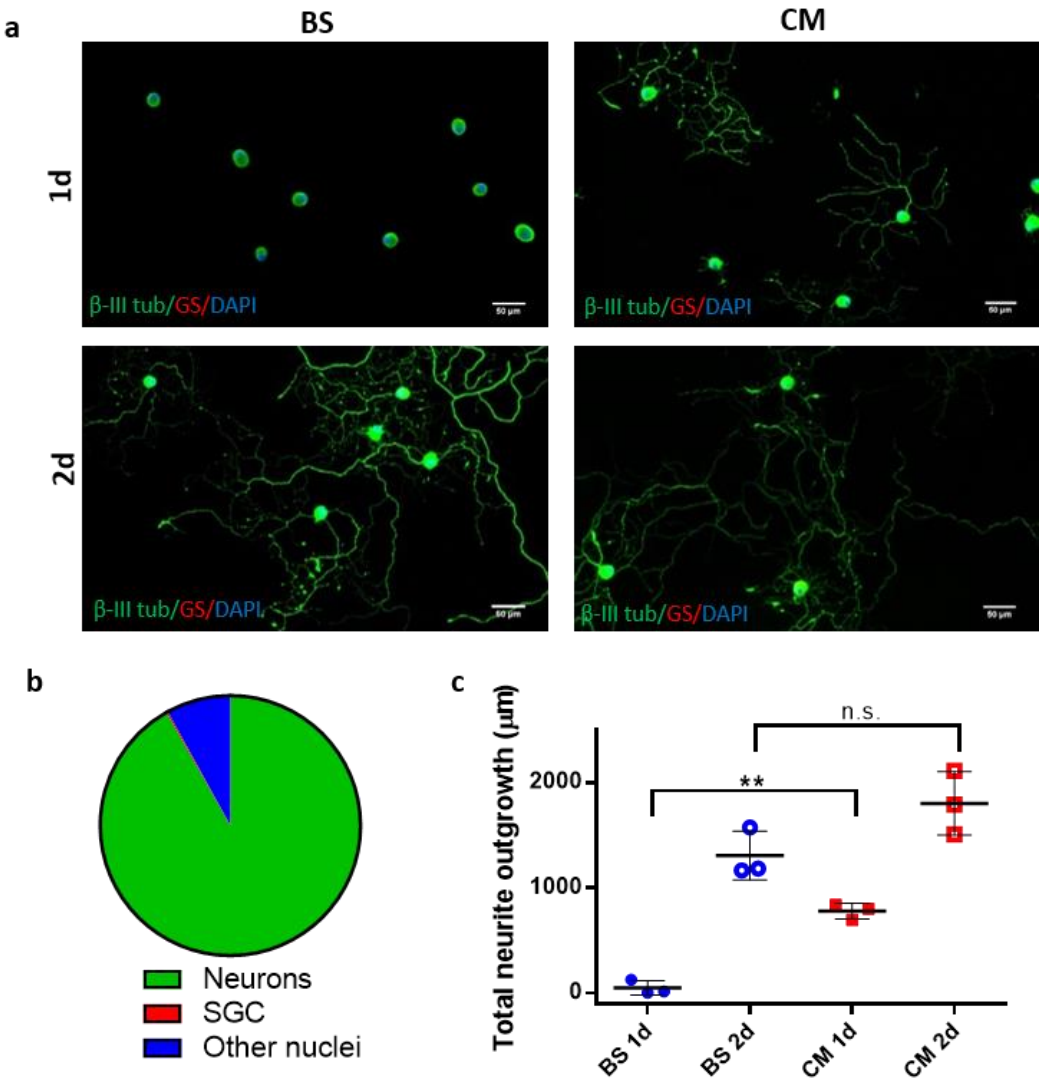


Figure 2.7 MACS purified DRG cultures respond to conditional medium (CM) taken from non-MACS purified cultures. a- *representative ICC of MACS purified neurons plated in BS or CM taken from non-MACS purified DRG cultures for 1 or 2 days. Stained with β-III pan-neuronal marker and glutamine synthetase (GS).* ***b-*** *Quantification of percentage of neurons (91.8% ± 5.6%) and SGC (0.2% ± 0.3%) in MACS purified cultures plated in CM.* ***c-*** *Total neurite outgrowth for MACS purified neurons in response to CM. One-way ANOVA, Tukey post hoc, p=0.0082, n=3 independent cultures.*

2.3.7 MACS purified neurons are responsive to NGF

Based on the CM experiments MACS purified neurons are responsive to secreted factors, one of the growth factors that neuronal and non-neuronal cells are known to secrete is NGF. Therefore, we further investigated whether MACS purified cells response to NGF specifically. Purified neurons were exposed to increasing NGF concentrations and neurite outgrowth was measured. Compared to B27 control medium treatment with 10 ($p=0.004$), 50 ($p<0.0001$) and 100ng/ml ($p=0.005$) NGF showed a significant increase in neurite outgrowth, one-way ANOVA with Tukey post-hoc. B27 control 19 ± 9µm, 1ng/ml NGF 82 ± 7µm, 5ng/ml NGF 389 ± 226 µm, 10ng/ml NGF 824 ± 131 µm, 50ng/ml NGF 977 ± 229 µm and 100ng/ml NGF 800 ± 28 µm (**Fig 2.8a and b**). From this an optimal concentration of 10ng/ml was derived and used in all further experiments involving NGF treatment. Additional neurite outgrowth measurements showed the same trend with minor exceptions. The number of neurites per neuron ($p=0.0192$) and the total length of neurites from the longest neurite branch ($p=0.0332$) also showed a significant increase with 5ng/ml NGF and treatment of MACS purified neurons with 50ng/ml failed to show any significant difference with the shortest neurite parameter (**Supplementary Fig 2.4**).

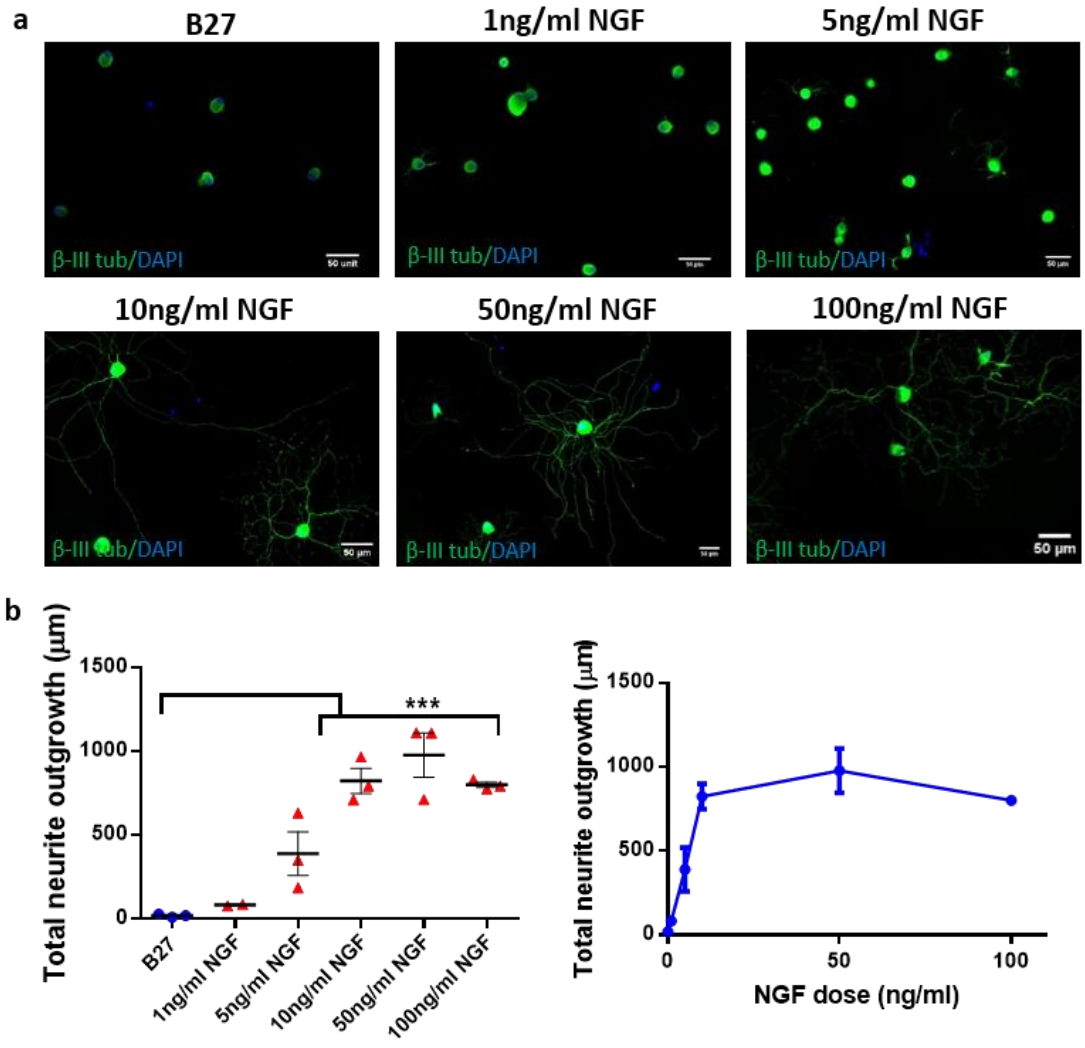


Figure 2.8 MACS purified neurons respond to NGF in a dose dependent manner. a- *Representative ICC of MACS purified neurons treated with increasing concentrations of NGF. Neurons are stained with pan-neuronal marker β-III tubulin.* ***b-*** *Quantification of total neurite outgrowth for cells treated with NGF and dose response curve of total neurite outgrowth in response to NGF. One-way ANOVA with Tukey post hoc, B27 vs 10ng/ml NGF p=0.004 B27 vs 50ng/ml NGF p<0.0001 and B27 vs 100ng/ml NGF p=0.005, n=2-3 individual experiments.*

2.4 Discussion

2.4.1 Benefits of MACS compared to other methods of purification

As described MACS purification is efficient in enriching and purifying cultures of both non-neuronal cell cultures and neuronal cultures. Compared to other methods such as immunopanning or FACS, MACS purification is a faster method taking only a few hours from tissue to culture compared to days of preparation like immunopanning for which plates for negative and positive panning are prepared one day in advance plus at least a day to grow the culture or several hours of processing time to isolate specific cells using FACS (Miltenyi et al. 1990). In some cases, faster methods require the use of less reagents such as buffers and culture medium and so have the advantage of being cheaper and less labour intensive. Immunopanning, for example, requires a large amount of antibodies to coat the panning plates in addition to buffers and culture medium making this experiment costlier than MACS purification. However, we found that when using MACS purification of DRGs that there is one confounding element which makes this method less cost effective. Due to the loss of medium and large diameter neurons there was a decrease in the yield of total neurons which therefore for larger experiments results in the need for a greater number of animals to collect DRGs from, which is more expensive.

The advantage of the MACS technology amongst others is that it is an adaptive technique. For example, Schwarz *et al* used the MACS LD column with anti-myelin beads in order to clean up hippocampal tissue samples analysing it by FACS (Schwarz and Bilbo 2013). Lee *et al* also used MACS as a prior step to FACS in order to purify CD11b/c positive cells from brain tissue before FACS analysis (J. Lee et al. 2008). Furthermore, development of MACS purification has been inspired by some of the more traditional purification methods such as immunopanning. Nguyen *et al* describes how they used the mouse monoclonal p75 antibody, that had formally

been commonly used in immunopanning, in combination with MACS microbeads and ran samples of neocortex through the MACS column to purify p75 positive cells. In this study, two separate populations of cells from the neocortex were collected, flow through from the MACS column contained all p75 negative cells (cortical plate cells) and cells washed off the column contained the p75 positive fraction of cells (subplate neurons) (V. Nguyen and McQuillen 2010).

Feldmann *et al* describes a novel protocol adapting MACS technology to purify multiple cell types from the same tissue sample, an improvement on the current methods which would require a separate tissue sample for the isolation of each specific cell population of interest. Brain samples from postnatal mice were collected, digested and then dissociated using MACS dissociation enzymes and gentleMACS dissociator followed by the removal of myelin using anti-myelin microbeads. An anti-ACSA-2 antibody was then used to tag astrocytes in the cell suspension before running it through a MACS LS column. Astrocytes were washed off the column and used for culturing. However, the flow through from the column was incubated with the non-neuronal cell antibody cocktail and anti-biotin microbeads in order to deplete non-neuronal cells by running the sample through a MACS MS column. Using this method, cultures of 92% astrocytes and 86% neurons were obtained (Feldmann et al. 2014). This study is an excellent example for how versatile this technique is.

In conclusion, there are many techniques that can be used to purify cells that is beneficial for the investigation of mechanisms and the roles specific cell types play in a specific disease state, such as pain. Here, we have shown that MACS purification of DRG cultures can effectively be used to further investigate specific behaviours and responses of sensory neurons. Our group already published a successful application of the MACS technique where DRG neurons were purified using MACS and RNA was isolated and prepared for RNA sequencing. This study identified specific genes that were differentially expressed in neuronal and non-neuronal cells

(Thakur et al. 2014). However, there are limitations associated with this technique, such as the inability to measure changes in cell surface markers, which the use of MACS purified cultures may be better suited to address.

2.4.2 MACS purification effectively generates 90% pure sensory neuron cultures from whole DRG

Magnetic assisted cell sorting (MACS) has been widely used in the immunology field to isolate specific populations of immune cells. However, it is now also being used in the neuroscience field. Here we used MACS technology from Miltenyi biotech to isolate pure neuronal cultures from DRG and established that MACS purification of DRG results in viable cultures of 90% neuronal cells, a greater purity of neurons compared to other methods of purification such as immunopanning (X.-M. Zhang et al. 2010). However, contrary to non-MACS purified cultures, MACS purified neurons fail to adhere to coverslips coated in PLL and laminin. They also don't adhere to coverslips coated with collagen or concanavallin A. Our work shows that the optimal coating is a thin layer of matrigel on PLL coated glass coverslips or the plastic of 4-well plates.

It has previously been shown that different coatings result in different growth responses for neurons, fibroblasts and Schwann cells indicating that there could be a preference for a certain glycoprotein in the extracellular matrix (ECM) for each cell type. The ECM in the periphery, particularly during regeneration following injury is known to contain a number of components including laminin, fibronectin, collagen and other growth promoting elements (Ma et al. 2013). Evercooren *et al* tested the growth promoting role of laminin, fibronectin and collagen in DRG cultures from 9-10 week old human foetuses. They found that with collagen, the addition of NGF increased the success of adhesion of explants to coated plates. Compared to collagen, explants plated onto either fibronectin or laminin promoted a greater degree of neurite outgrowth, with laminin having the greater effect overall. Different coatings also showed

different patterns of neurite outgrowth: collagen showed a radial pattern and earlier migration of fibroblasts compared to Schwann cells, fibronectin enabled neurons to rapidly grow with more lateral branching patterns onto heavy layers of fibroblasts and more extensive Schwann cell migration and finally laminin showed no major radial bundles and instead exhibited an intricate branching pattern in all directions. Contrary to the other coatings, fibroblast migration was reduced although Schwann cells were still able to migrate (Baron-Van Evercooren et al. 1982). Differences between the neurite outgrowth has also been observed between central and peripheral neurons on different coatings. Compared to PLL coating there was rapid elongation of neurites from both peripheral and central neurons grown on laminin, however only peripheral neurons were shown to exhibit neurite elongation on fibronectin (Rogers et al. 1983). McGarvey *et al* describes how different coating affect the growth of Schwann cells. Schwann cells are known to release components of the extracellular matrix including fibronectin, laminin and collagen in culture, and despite the fact that the addition of fibronectin enhances the growth of Schwann cells, Schwann cells grown on collagen coated coverslips in the presence of laminin grew with a 2-6 fold higher adhesion rate compared to fibronectin (McGarvey et al. 1984). It is also known that there are a number of integrin receptors expressed on neuronal and non-neuronal cells which facilitate binding to glycoproteins. Neurons have been shown to express for instance tissue specific integrins, such as α7β1 integrin, which is laminin binding and predominately expressed in DRGs and α5β1 as well as α4β1, which is fibronectin binding and expressed in sensory neurons (Tomaselli et al. 1993). Since different cell types bind and grow with different affinities to different glycoproteins it is reasonable to assume that that different coatings will preferentially bind different cell types.

It is unknown whether the reason that MACS neurons do not bind to laminin or other tested substrates is due to a lack of adhesion molecules which could be shredded from neuronal cells

as they are passed down the MACS LD column or if it is the result of the lack of the non-neuronal cells. Preferential binding of a non-neuronal cell type such as a SGC for example could act as an anchor point for the adhesion of neuronal cells. A study by Ng *et al* that described a method of purifying cultures of SGC which takes advantage of a loose association of neurons in culture. DRG cultures were plated onto poly-DL-ornithine overnight after which the loosely attached neurons in suspension were removed and re-plated, leaving the more strongly attached SGCs attached to the base of the plate. Purity of the neurons was said to be 70% neuronal (Ng et al. 2013). This method supports the hypothesis that SGC binding first in culture could act as an anchor point for neuronal cells which are found in close association with SGC in culture (Ng et al. 2013) as culturing overnight allows the SGC to bind before neurons attach at a later time point e.g. after 24 hours in culture.

Mock MACS purification of DRG neurons showed that the MACS LD column itself did not purify or cause any cell death. It produced cultures that had a normal distribution of small, medium and large cell diameter neurons. However, following MACS purification we observed that there was a loss in medium and large diameter neurons compared to non-MACS purified DRG cultures. We hypothesised that this was due to neurons being retained in the MACS LD column which has a pore size of 40 µm, and so we tested this by collecting three different fractions off the column including the MACS purified neurons, flow-through from the column where the MACS LD column was released from the magnetic field and the plunger fraction where any remaining cells were released using the plunger. As expected the majority of neurons were present in the MACS fraction and the flow-through and plunger fractions contained mainly SGCs. Surprisingly, the flow-through and plunger fraction combined accounted for only 23% of the total number of neurons collected from all fractions. Cell size distribution analysis revealed a shift towards small diameter neurons in neurons from the flow-through and the plunger fractions. We hypothesised that the loss of medium and large diameter neurons was due to

these larger cells being caught in the MACS LD column. However, we excluded this option when mock MACS purification showed no change in cell size distribution. The only difference between the mock MACS purification and the MACS purification procedure was the presence of MACS antibodies. We therefore believe that the complexed by the primary and secondary antibodies clog up the pores of the MACS LD column and restrict the passage of large diameter. Experience in the lab showed that no more than the DRGs of two adult mice can be passed through the MACS LD column otherwise there is a detrimental impact on the yield of neurons, where the purification of DRGs from 3 adult mice has a similar yield of neurons as the DRGs from 2 adult mice. Another reason for the lack of medium and large diameter neurons in the MACS cultures could be due to insufficient dissociation of neurons from SGCs which results in large aggregates. SGC greatly outnumber neuronal cells in the DRG and are known to remain associated with neurons in culture leading to various problems when enriching neuronal cultures (Ng et al. 2013). Medium and large diameter neurons have a higher probability to be associated with SGCs and therefore more likely to be insufficiently dissociated due to their increased surface area that binds a larger number of SGC compared to small diameter neurons.

One approach to test this hypothesis and potentially improve the purification is to use a stronger enzyme mix than the dispase and collagenase mix that we have been using. One possible enzyme, which is stronger than collagenase, is papain (Worthington Biochemical Corporation 2017). However, over-digestion bears the risk to kill the cells or destroy the epitopes that are needed for the binding of the antibodies that are used in the non-neuronal antibody cocktail for MACS purification. One other possibility would be to utilise the gentle MACS dissociator, which homogenises tissues using a set programme that is specific for each tissue. The MACS dissociator is used in a number of studies that also use MACS to subsequently purify cells (Kar et al. 2013; J. Lee et al. 2008; V. Nguyen and McQuillen 2010).

In addition to neurons being trapped in the MACS LD column, it is possible that decreased numbers of cells in the plunger fraction collected could also have been the result of a neurons being shredded when forced through the pores of the MACS LD column. There are a number of other DAPI positive nuclei in cultures following MACS purification and in cultures from flow-through and plunger fractions. Typically, MACS purified cultures contain a negliable number of SGCs, however, there is DAPI staining in these cultures which does not associate with neuronal markers. These other nuclei may be those of other cell types such as Schwann cells, however we rather believe that they are the nuclei of destroyed medium and large diameter neurons which might have been shredded in either the MACS LD column (particularly in the plunger fraction) or during the dissociation and titration procedure. Combined with the MACS protocol, the dissociation and titration could be too stressful for the larger neurons. This hypothesis could be tested by apoptotic assays, such as TUNEL staining which stains for DNA fragments. The same observation of DAPI stained nuclei present in post-MACS purified cultures that do not associate with markers for their cell type was made by Feldmann *et al* where after purifying astrocytes or neurons they observed evidence of contamination of DAPI stained nuclei that did not stain positive for neurons or astrocytes nor for other cell markers GFAP or MAP2 (Feldmann et al. 2014).

After establishing that MACS purification was effective in generating pure neuronal cultures we then used a neurite outgrowth assay to establish if these cultures behaved in a similar manner to non-MACS purified cultures. We observed that MACS purified neurons exhibited a delay in neurite outgrowth after 24 hours in culture compared to non-MACS purified cultures. However, after 48 hours there was a significant increase in neurite outgrowth in MACS purified cultures compared to 24 hours where the neurite outgrowth in MACS purified cultures after 48 hours was similar to neurite outgrowth of non-MACS purified cultures after 24 hours in culture. This initial lag in neurite outgrowth for 24 hours in MACS purified cultures before a

significant increase in neurite outgrowth after 48 hours in culture was also observed in cultures grown in different culture mediums as there was no significant difference in neurite outgrowth with different mediums at either 24 or 48 hours in culture. We hypothesised that MACS purified cultures are initially inhibited for neurite outgrowth since there is a lack of cell contact or secreted factors from non-neuronal cells. Our experiments with the conditioned medium of non-MACS purified cultures on MACS purified cultures showed that MACS purified neurons are responsive to secreted factors in the conditioned medium, supporting the hypothesis that the lag in neurite outgrowth is due to the lack of secreted factors from non-neuronal cells. This lag in neurite outgrowth following purification has also been observed by Ng *et al.* Here, DRG neurons were purified by repeated plating; the dissociated DRG cell suspension without purification is plated and left to adhere overnight before the loosely attached neurons are removed from the culture and re-plated the next day. This results in an enrichment to 70% neurons in the culture. Subpopulations of neurons were then isolated using MACS. First, IB4 (non-peptidergic nociceptors) cells were tagged and retained in the MACS column and IB4 negative cells were collected in the eluate, along with any remaining non-neuronal cells. Both IB4 positive and negative fractions were cultured. Cultures of IB4 negative neurons plus non-neuronal cells were tested for neurite outgrowth after 24 hours in culture and cultures of IB4 positive neurons were tested for neurite outgrowth after 48 hours in culture. Both cultures showed the same amount of neurite outgrowth suggesting that in the absence of non-neuronal cells there is a delay in neurite outgrowth (Ng et al. 2013).

Knowing that MACS purified neurons responded to secreted factors we further showed that they also specifically responded to NGF which was able to induce neurite outgrowth by itself. Our results show that the optimal dose is 50 ng/ml of NGF. 10 ng/ml did induce neurite outgrowth, but not to the extent that 50 ng/ml did. 100 ng/ml NGF also induced neurite outgrowth but showed a decreased potency to induce neurite outgrowth compared to 50

ng/ml. This might indicate saturation of the receptors; however, this would not fully explain a decrease in neurite outgrowth following 100 ng/ml compared to 50 ng/ml. It is possible that high concentrations of NGF are toxic to cultures, this could be tested by increasing the concentration of NGF used to treat MACS purified cultures. One other explanation could be that the vehicle used for NGF is toxic in large doses. This can be tested by treating MACS purified with vehicle alone, however this is unlikely to be the case as NGF vehicle used contains 0.1% BSA in HBSS which is diluted 1:10 000 in culture medium prior to plating for 10 ng/ml NGF. As already described (chapter 1.1) the high affinity NGF receptor TrkA binds NGF in its monomeric form via its extracellular ligand binding domain before it then forms a dimer with another unbound TrkA. Higher NGF concentrations could result in all TrkA monomers binding NGF and no empty monomers available for dimerization which leads to saturation. The effect of 10 ng/ml NGF proved itself valuable to us since it increases neurite outgrowth, however, it allowed room for improvement. Therefore, for all subsequent experiments we used 10 ng/ml NGF 10 ng/ml NGF, which is close to 0.1 nM, the EC50 for NGF as tested in PC12 cells (Mahapatra et al. 2009), as a positive control for producing significant neurite outgrowth compared to MACS purified cultures cultured in the absence of NGF for 24 hours which exhibit little or no neurite outgrowth.

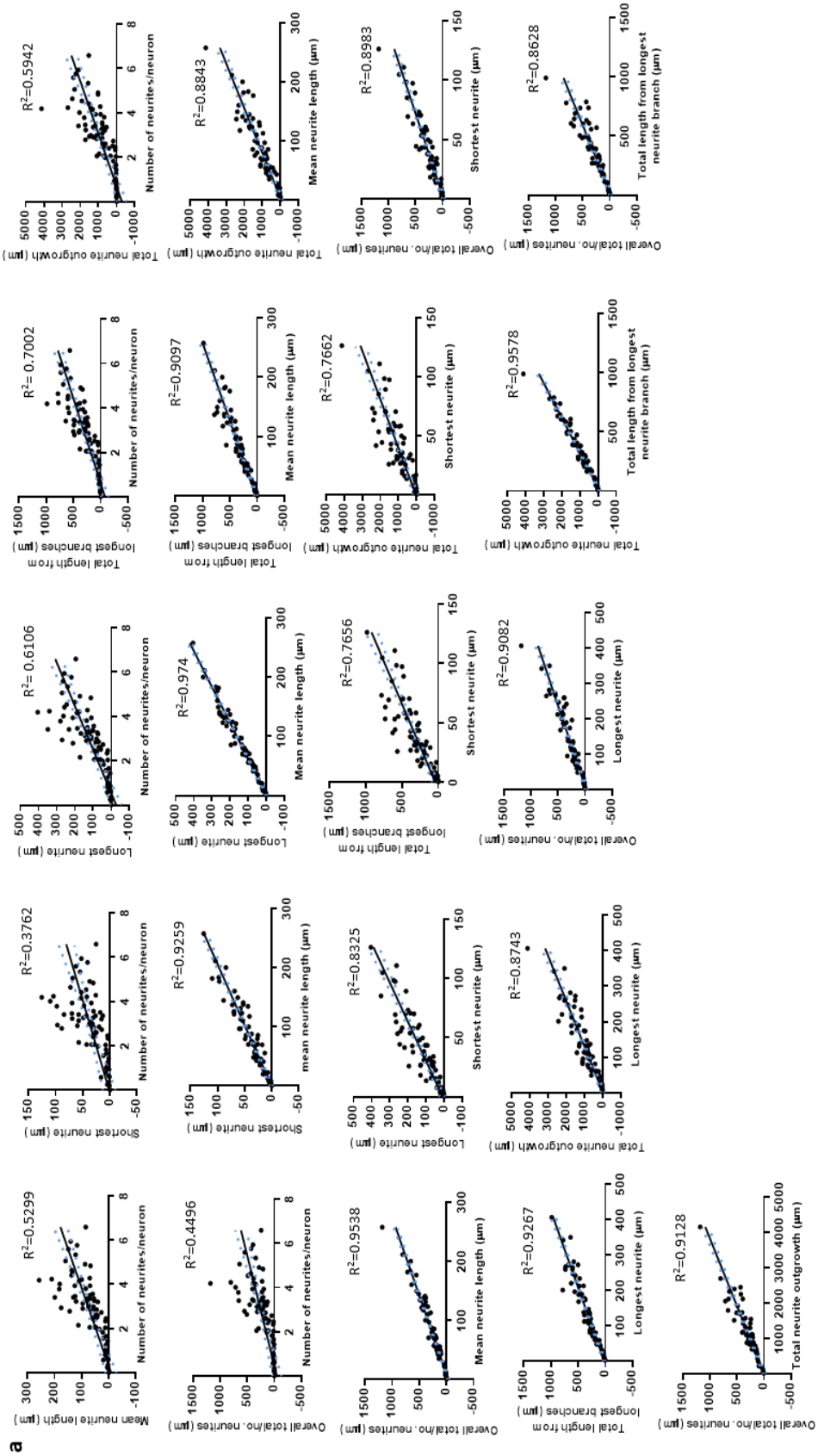
2.4.3 Neurite outgrowth as a measurement of neuronal responses

Neurite outgrowth is a commonly used assay in order to measure neuronal responses to treatment with growth factors *in vitro* (Rogers et al. 1983). We adapted this in our study and optimised a neurite outgrowth assay to study the effect of MACS purification on neurons. There have been a number of methods created to semi-automate or automate this technique, all of them related to ImageJ, such as the an automated plugin NeuriteTracer (Pool et al. 2008), the Neurite-J plugin which can be used to measure neurite outgrowth from organotypic cultures using a method similar to the Scholl analysis of concentric circles (Torres-Espín et al.

2014) and the semi-automated plugin NeuronJ. Upon testing both the NeuronJ and the NeuriteJ plugin, we found that they both rely on specific immunostaining of neurons. Also, upon closer observation of the created images, we noticed that for a significant proportion of neurons there are small neurites branching off from the main branches which these plugins are not sensitive enough to detect. Furthermore, in cultures treated with NGF for 24 or 48 hours or longer there were many neurites which crossed over in areas where neuronal bodies grew in patches which the plugins were not able to separate in single neurites or neuronal bodies. We therefore chose not to use the time saving ImageJ plugin. The initial characterisation was performed by manual tracing neurites of the neurites using Fiji – an updated version of ImageJ. Using this method also allowed the freedom to acquire a number of different growth parameters for each neuron analysed.

In conclusion, we have characterised here a method for purifying DRG neuronal cultures using MACS. This method can be used for a number of potential applications ranging from the use of isolated neuronal cultures, to the introduction of purified neurons into co-cultures. Co-cultures can be then used to study the interaction of neurons with other cells such as immune cells in neuroimmune interactions. Isolated neuronal cultures however have the benefit of being able to be used to distinguish the direct effect of pain mediators, such as NGF, on the neurons directly vs the indirect of NGF via other cells present in non-MACS cultures. This distinction between the direct vs the indirect effect of pain mediators is a confound in a significant proportion of published work, which in the future could be addressed using MACS technology.

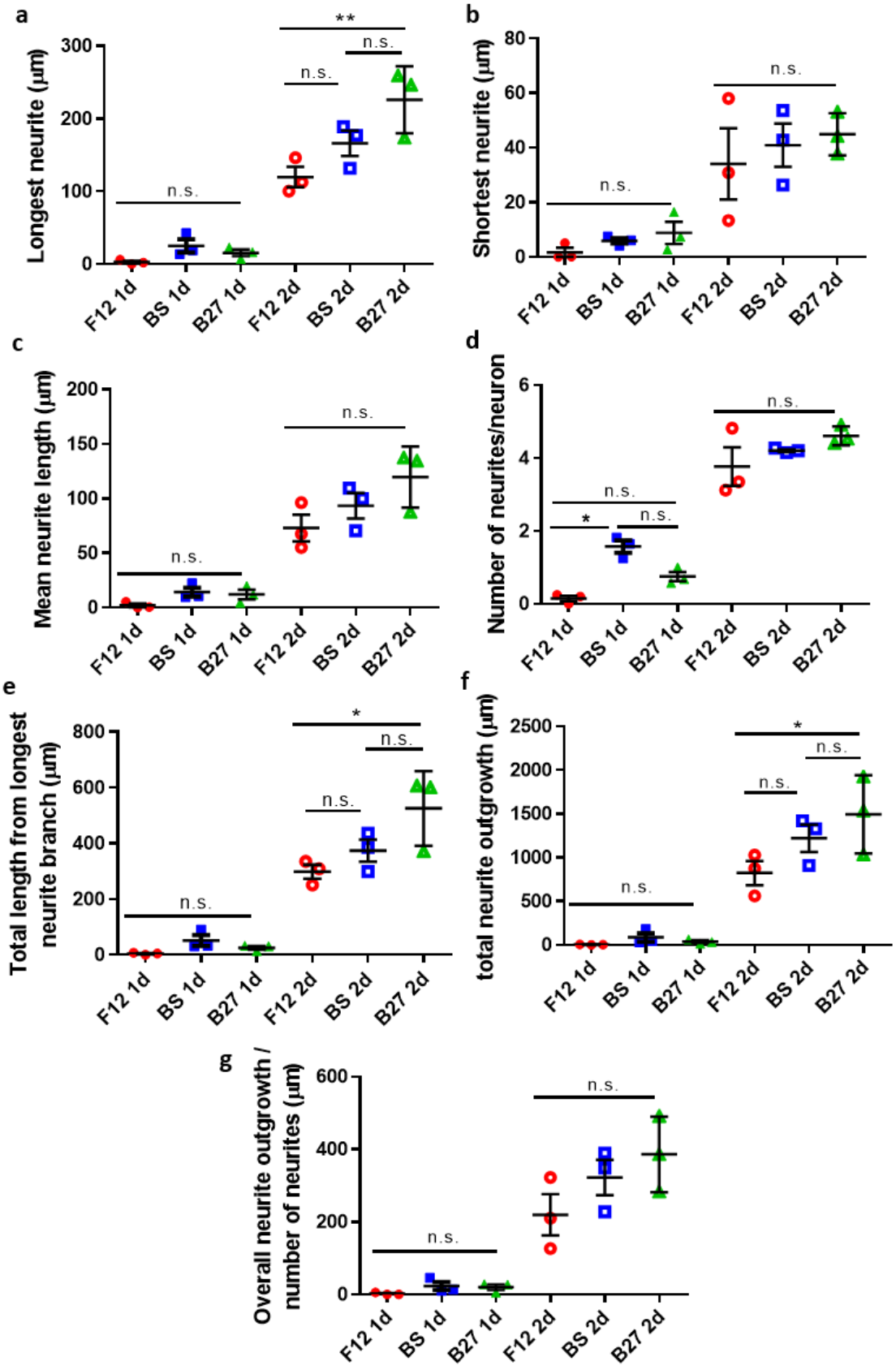
2.5 Supplementary material



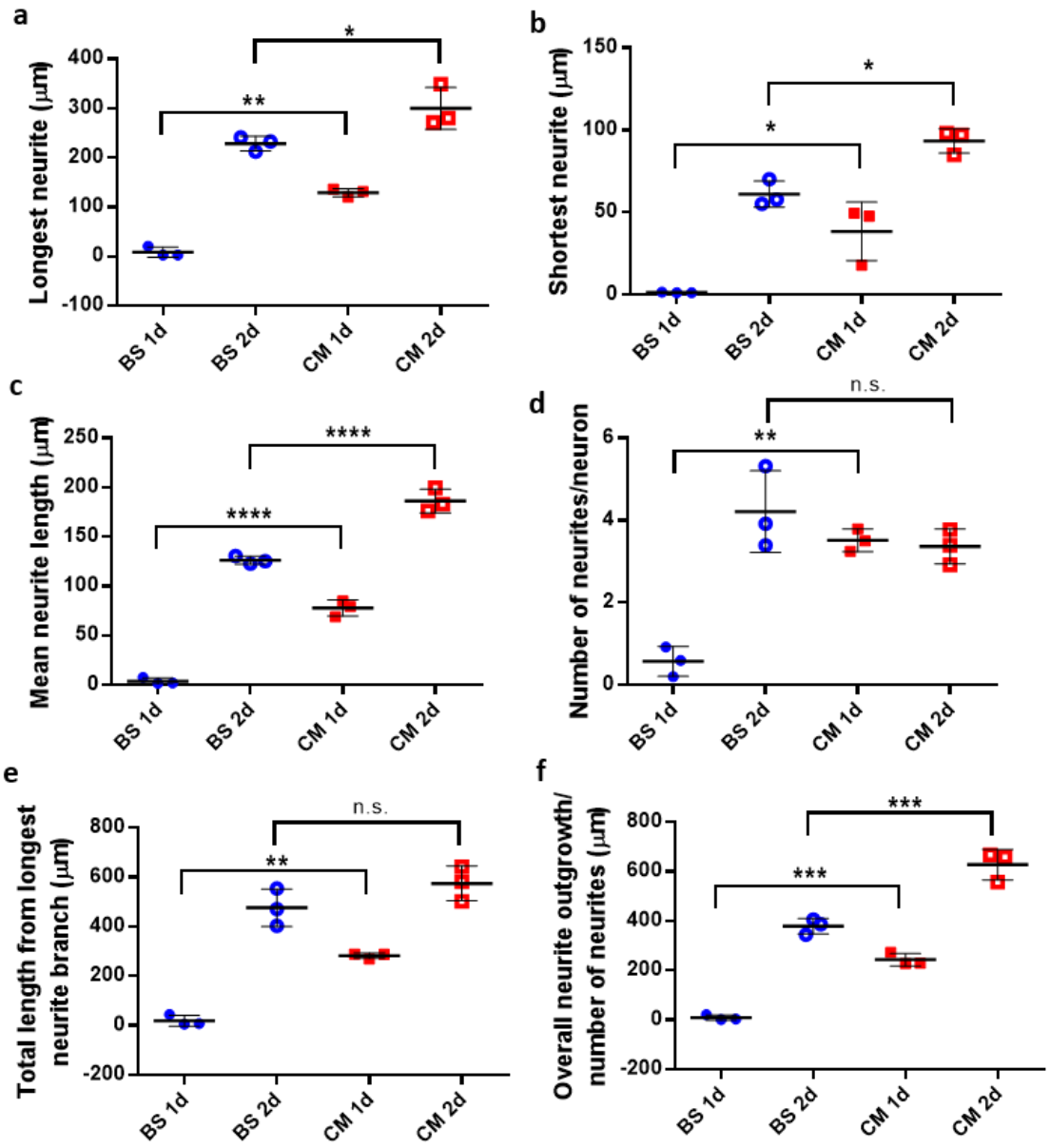
Supplementary figure 2.1 Linear regression comparison of neurite outgrowth measurements. *R^2 value is calculated from the line of best fit (solid blue line) and indicated for each comparison. Dotted blue lines show the 95% CI for the line of best fit.*

Comparison			R^2	95% CI of slope
Mean neurite length (µm)	vs	Longest neurite (µm)	0.974	1.583 to 1.708
Total neurite length from longest branches (µm)	vs	Total neurite outgrowth (µm)	0.9578	3.236 to 3.569
Mean neurite length (µm)	vs	Overall total / number of neurites (µm)	0.9538	3.579 to 3.966
Longest neurite (µm)	vs	Total neurite length from longest branches (µm)	0.9267	2.175 to 2.48
Mean neurite length (µm)	vs	Shortest neurite (µm)	0.9259	0.453 to 0.517
Total neurite outgrowth (µm)	vs	Overall total / number of neurites (µm)	0.9128	0.2443 to 0.2823
Mean neurite length (µm)	vs	Total neurite length from longest branches (µm)	0.9097	3.562 to 4.127
Longest neurite (µm)	vs	Overall total / number of neurites (µm)	0.9082	2.044 to 2.372
Shortest neurite (µm)	vs	Overall total / number of neurites (µm)	0.8983	6.694 to 7.834
Mean neurite length (µm)	vs	Total neurite outgrowth (µm)	0.8843	12.07 to 14.29
Longest neurite (µm)	vs	Total neurite outgrowth (µm)	0.8743	7.164 to 8.554
Total neurite length from longest branches (µm)	vs	Overall total / number of neurites (µm)	0.8628	0.8073 to 0.9729
Shortest neurite (µm)	vs	Longest neurite (µm)	0.8325	2.703 to 3.334
Shortest neurite (µm)	vs	Total neurite outgrowth (µm)	0.7662	21.2 to 27.47
Shortest neurite (µm)	vs	Total neurite length from longest branches (µm)	0.7656	6.095 to 7.901
Number of neurites/neuron	vs	Total neurite length from longest branches (µm)	0.7002	111.8 to 152
Number of neurites/neuron	vs	Longest neurite (µm)	0.6106	41.45 to 60.44
Number of neurites/neuron	vs	Total neurite outgrowth (µm)	0.5942	341 to 503.9
Number of neurites/neuron	vs	Mean neurite length (µm)	0.5299	22.21 to 34.72
Number of neurites/neuron	vs	Overall total / number of neurites (µm)	0.4496	75.14 to 127.4
Number of neurites/neuron	vs	Shortest neurite (µm)	0.3762	8.457 to 15.72

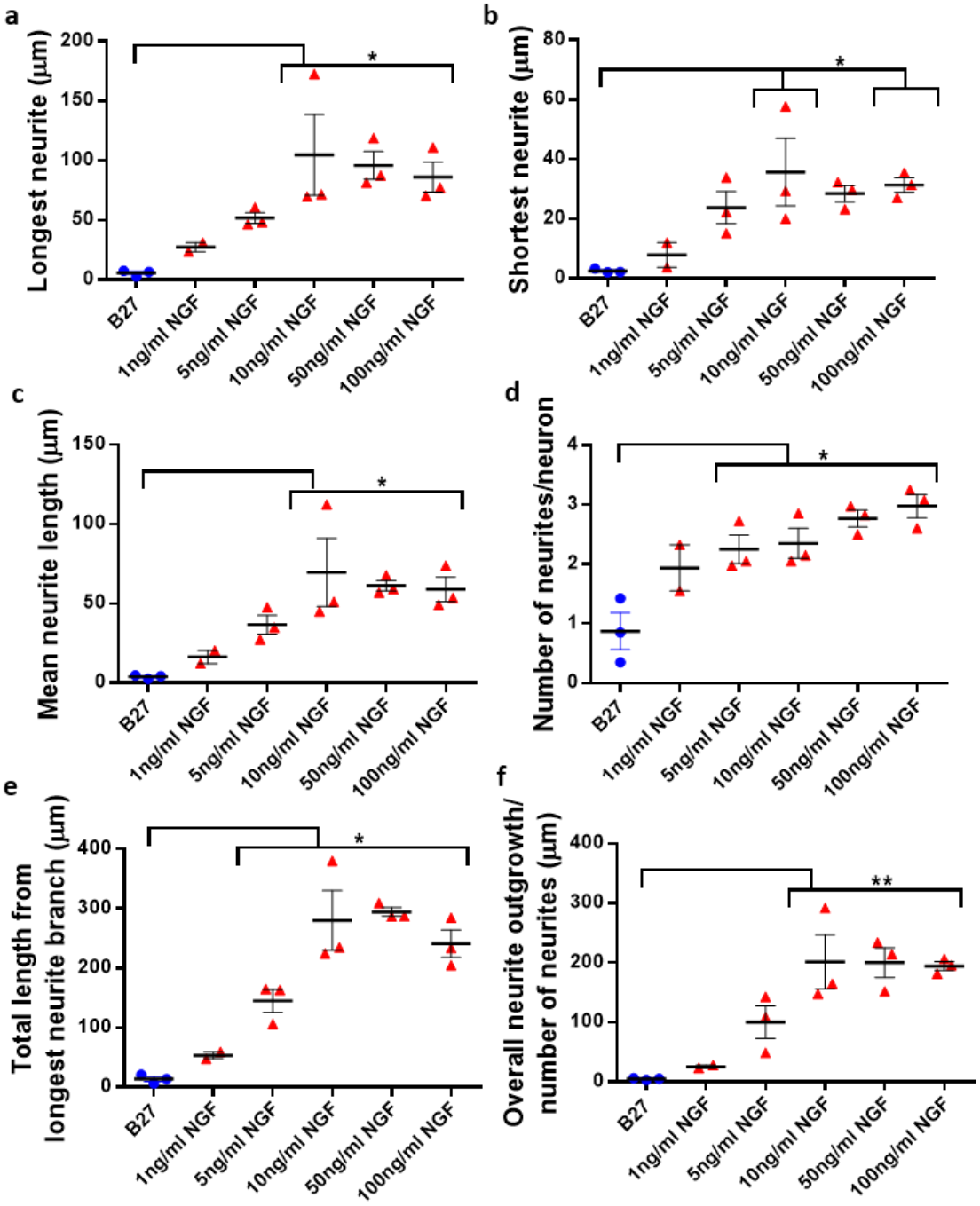
Supplementary table 2.1 Comparison of different neurite outgrowth parameters. *Highlighted in yellow are parameters that show a significant positive correlation.*


a
Longest neurite (µm)
300
200
100
0
**
n.s.
n.s.
n.s.
F12 1d
BS 1d
B27 1d
F12 2d
BS 2d
B27 2d
b
Shortest neurite (µm)
80
60
40
20
0
n.s.
n.s.
c
Mean neurite length (µm)
200
150
100
50
0
n.s.
n.s.
d
Number of neurites/neuron
6
4
2
0
n.s.
*
n.s.
n.s.
e
Total length from longest neurite branch (µm)
800
600
400
200
0
*
n.s.
n.s.
n.s.
f
total neurite outgrowth (µm)
2500
2000
1500
1000
500
0
*
n.s.
n.s.
n.s.
g
Overall neurite outgrowth / number of neurites (µm)
600
400
200
0
n.s.
n.s.

Supplementary figure 2.2 Additional neurite outgrowth measurements of MACS purified neurons cultured for 1-2 days in different growth mediums. a *longest (p=0.0028)* ***b*** *shortest and* ***c*** *mean neurite length,* ***d*** *number of neurites (p=0.0139),* ***e*** *total neurite outgrowth from longest branch (p=0.014),* ***f*** *total neurite outgrowth (p=0.049) and* ***g*** *overall neurite outgrowth normalised to number of neurites. One-way ANOVA with Tukey post hoc, n=3 individual cultures.*



Supplementary figure 2.3 Additional neurite outgrowth measurements of MACS purified neurons treated with CM from non-MACS purified DRG cultures. ***a*** *longest (1d p=0.0011, 2d p=0.0240)* ***b*** *shortest (1d p=0.0103, 2d p=0.0216) and* ***c*** *mean neurite length (p<0.0001),* ***d*** *number of neurites (p=0.0013),* ***e*** *total neurite outgrowth from longest branch (p=0.0014) and* ***f*** *overall neurite outgrowth normalised to number of neurites (1d p=0.0003, 2d p=0.0002). One-way ANOVA with Tukey post hoc, n=3 individual cultures.*



Supplementary figure 2.4 Additional neurite outgrowth measurements for MACS purified neurons stimulated with increasing concentrations of NGF*. **a** longest (B27 vs 10ng/ml NGF p=0.0124, 50ng/ml NGF p=0.0226, 100ng/ml NGF p=0.0446) **b** shortest (B27 vs 10ng/ml NGF p=0.0156, 100ng/ml NGF p=0.0375) and **c** mean neurite length (B27 vs 10ng/ml NGF p=0.0081, 50ng/ml NGF p=0.0202, 100ng/ml NGF p=0.0261) , **d** number of neurites (B27 vs 5ng/ml NGF*

p=0.0192, 10ng/ml NGF p=0.0121, 50ng/ml NGF p=0.0019, 100ng/ml NGF p=0.0008), ***e*** *total neurite outgrowth from longest branch (B27 vs 5ng/ml NGF p=0.0332, 10ng/ml NGF p=0.0001, 50ng/ml NGF p<0.0001, 100ng/ml NGF p=0.0006) and* ***f*** *overall neurite outgrowth normalised to number of neurites (B27 vs 10ng/ml NGF p=0.0019, 50ng/ml NGF p=0.0020, 100ng/ml NGF p=0.0025). One-way ANOVA with Tukey post hoc, n=2-3 individual cultures.*

Chapter 3: Knockout of TrkA or p75 using viral vectors

3.1 Introduction

As discussed previously (chapter 1.2), investigations into the role of NGF in pain have previously used global knockout mice for either NGF, TrkA or p75. These mice have defects in the development of sensory neurons and are hyposensitive to thermal and mechanical stimuli in addition to a shortened lifespan; global TrkA and NGF knockout mice do not survive past weaning (Bergmann et al. 1997; K. F. Lee et al. 1992; Crowley et al. 1994; Smeyne et al. 1994). In order to circumvent these survival issues we obtained from the international mouse phenotyping consortium (IMPC) (IMPC 2017c; Bradley et al. 2012) floxed mouse lines for the conditional knockout of the *NTRK1* and *NGFR* genes, encoding the NGF receptors TrkA and p75, respectively. The IMPC aims to generate commercially available mouse lines with mutations for targeted knockout of 20 000 known mouse genes and the *NTRK1* and *NGFR* floxed mice are two of them (Bradley et al. 2012; IMPC 2017c). My hope was that these two lines when used in combination with the previously described MACS purification (chapter 2) would shed light on the role of NGF on neurons alone *in vitro*.

3.1.1 Floxed *NTRK1* or *NGFR* animals for targeted knockout of NGF receptors

The *NTRK1* fl/fl and *NGFR* fl/fl mouse lines were generated using embryonic stem (ES) cell clones. Bradley *et al* described the protocol that was used. LoxP sites flank the critical region of the gene of interest (common to all isoforms of the gene). This critical region was determined

as being within the first 50% of the coding gene where deletion of the floxed regions results in a frame shift generating a truncated non-functional protein or mutant mRNA that likely to be degraded (Bradley et al. 2012; Hofker and van Deursen 2011). Targeting vectors used contained a lacZ reporter gene linked to an internal ribosome entry site (IRES) which allows independent transcription of the marker protein upstream of a neomycin cassette under the control of the hbactP promoter, the autonomous human β-actin promoter. This whole complex of the lacZ reporter and neomycin cassette were flanked by flippase recognition target (FRT) sites and followed by Lox P sites located in intron regions upstream and downstream of a critical exon region from the gene of interest (**Fig 3.1**) (Skarnes et al. 2011). For Cre mediated excision the LoxP sites are usually placed 250 base pairs (bp) away from the exons to avoid interference with the splicing process (Hofker and van Deursen 2011; Skarnes et al. 2011). Targeting vectors are then transfected into the ES cells using electroporation where the wildtype copy of the allele is replaced by the targeting vector via homologous recombination. ES cells are then screened for successful recombination via neomycin resistance. Selected ES cells were then injected into blastocytes and implanted into surrogate mothers that generate mutant offspring (Bradley et al. 2012; Hofker and van Deursen 2011; Branda and Dymecki 2004). These offspring, born within the IMPC project were then bred in order to obtain different knockout strains (**Fig 3.1**) (Skarnes et al. 2011). The first litter of mice are referred to as the tm1a strain which contain the targeting vector and therefore are reporter tagged mice (lacZ) with conditional potential for gene knockout. Crossing of tm1a mice with a Cre line results in excision of the loxP flanked region and therefore knockout of the gene of interest, however expression of the lacZ reporter remains in the resulting tm1b line. The tm1c line can also be generated from the original tm1a mice through crossing of this line with a Flp mouse line which results in excision of the targeting vector region between the FRT flanked regions of both the neomycin and the lacZ genes, thus generating a conditional

knockout mouse line without the lacZ reporter. Crossing of the tm1c line with a Cre line results in the tm1d line which exhibit knockout of the gene of interest in the absence of the lacZ reporter (Skarnes et al. 2011; IMPC 2017c).

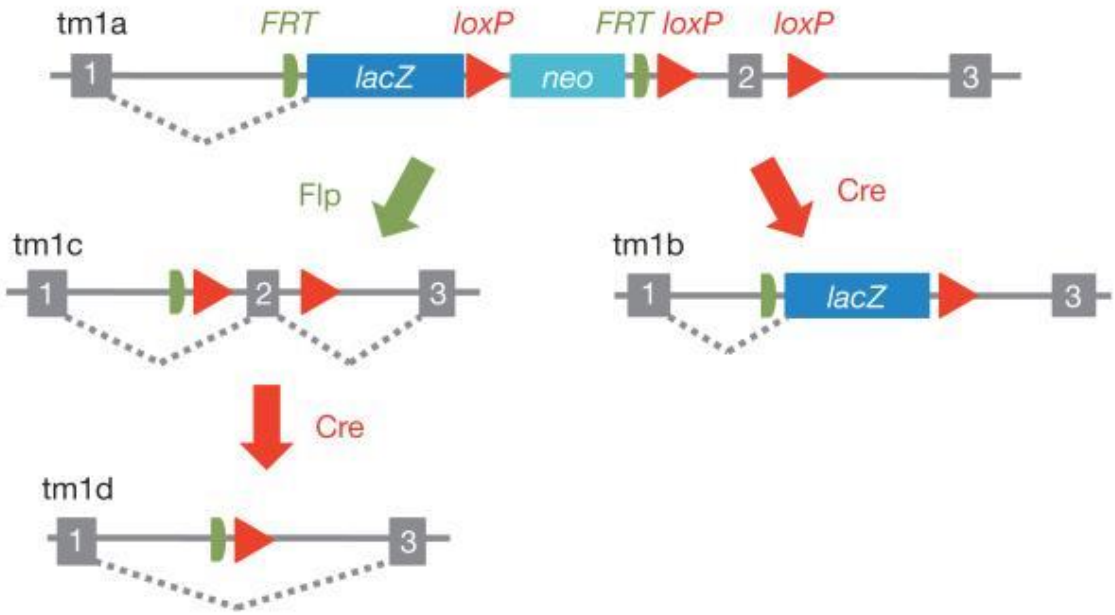


Figure 3.1 Schematic showing the generation of conditional knockout mouse lines from the IMPC. *Schematic taken from Skarnes et al describes generation of floxed mice of specific genes starting from tm1a conditional knockout mouse lines with a lacZ reporter. Mice are crossed either with a Cre recombinase mouse to generate tm1b knockout mice with a reporter gene or generate tm1c conditional knockout mice without the reporter gene following crossing with a FLP recombinase mouse. Finally, tm1c mice can be crossed with a cre expressing mouse to generate tm1b knockout mice without a reporter.*

In this study we obtained *NTRK1* fl/fl and *NGFR* fl/fl mouse lines stemming from the tm1c line from Harwell UK as part of the IMPC. Hence they are conditional knockout models based on the presence of Cre recombinase. In the *NTRK1* fl/fl mouse line the loxP sites flank exons 4-6. Knockout of this section disrupts the expression of the high affinity NGF receptor TrkA.

However, expression of a truncated form of the protein from exons 1-3 is possible. Exons 1-3 of the TrkA protein encode part of the extracellular domain including 2 tyrosine protein kinase domains from the neurotrophic receptors and two of the four leucine rich repeat regions (Ensembl 2016b) As this truncated protein is non-ligand binding it would not be capable of interacting with endogenous NGF (Wiesmann et al. 1999). In addition to breeding of the mouse lines, the IMPC also publishes preliminary behavioural observations from tested mice (IMPC 2017e). Correlating with previous studies using *NTRK1* knockout mice (Smeyne et al. 1994), the tm1b version of gene knockout, where there is knockout from birth and the lacZ reporter present, there was evidence of pre-weaning lethality. A range of biochemical and behavioural tests were also run on the tm1b animals including monitoring blood chemistry, glucose tolerance, grip strength and open field tests however there was no significant difference observed compared to WT littermate controls. It is important to note that none of the tests used involved measuring of sensitivity to non-noxious or noxious stimuli and there is no data available for behavioural testing on tm1a or tm1c animals (IMPC 2017b). The *NGFR* fl/fl tm1c mouse contains loxP sites flanking exon 2. Knockout of exon 2 ablates expression of the *NGFR* gene and the protein it is encoding, the low affinity p75 neurotrophin receptor (IMPC 2017d). Upstream of the loxP site, exon 1 remains. However it is unlikely that this would produce any truncated protein expression as exon 1 is mainly non-coding. Also, a potential truncated protein would not contain any of the main extracellular signalling regions (Ensembl 2016a). Contrary with the literature on global p75 knockout mice where premature death at six months is observed (K. F. Lee et al. 1992; Bergmann et al. 1997), the IMPC behavioural data for the tm1b knockout of the *NGFR* gene (global knockout of *NGFR* gene and expression of the lacZ), shows pre-weaning lethality. In *NGFR* tm1b animals however, a number of other anatomical and behavioural deficits were found to be significantly altered compared to controls, including abnormal gait, grip strength, decreased total body fat and bone mineral

content, abnormal digit morphology and tremors (IMPC 2017a). There is currently no data available for NGFR tm1a or tm1c animals.

3.1.2 The Cre-LoxP system is a useful tool for targeted knockout of floxed genes

There have been a number of models developed to obtain conditional knockout mouse lines. Two in particular are currently in use; the FLP/FRT and the Cre/Lox-P system. However, there are new models emerging such as the Cre homologue Dre which recognises roxP sites (Sajgo et al. 2014). The Flippase recombinase (FLP) system involves cleavage of FRT sites by the bacteriophage enzyme FLP (Hofker and van Deursen 2011). The Cre recombinase is a bacteriophage P1 enzyme with both lytic and lysogenic phases in its life cycle (Hoess, Ziese, and Sternberg 1982; Leone et al. 2003) which catalyses recombination of DNA between loxP sites (Hoess, Ziese, and Sternberg 1982; Hoess, Wierzbicki, and Abremski 1986; Hofker and van Deursen 2011; S. Feil, Valtcheva, and Feil 2009). Four different lox (locus of crossing) sites have been identified, loxP, loxB, loxL and loxR , of which loxP was the most efficient due to symmetry and spacing between the sites (Hoess, Ziese, and Sternberg 1982) and has been widely used in later studies. LoxP sites are 34 bp long and consist of two homologous 13 bp regions which are inverted and separated by an 8 bp sequence (Hoess, Wierzbicki, and Abremski 1986). At normal body temperature of 37^{o}C Cre recombinase is substantially more efficient than FLP recombinase, therefore for *in vivo* studies the Cre/LoxP system is preferred (R. Feil et al. 1997).

3.1.3 Delivery of Cre recombinase using viral vectors

One method used to deliver Cre recombinase to specific cell types include the use of viral vectors to package and deliver virally encoded Cre. Here, we describe the use of lenti virus and adeno-associated virus, both of which are highly versatile tools.

3.1.3.1 Lenti-virus

Lenti virus (LV) delivered transgenes have the ability to integrate into both dividing and non-dividing cells, are non-pathogenic and can be stably expressed over several months (Heldt and Ressler 2009). LV can also be used to house large capacity genetic material, including large genes such as neurotransmitters or neuropeptides, and has been widely used to deliver both cell markers such as GFP and Cre recombinase into cells (Heldt and Ressler 2009; Gompf et al. 2015).

In addition to delivering Cre, LV have been used to knockout genes using other methods such as the use of siRNA. Zhang et al describes how small interfering RNA against p75 were packaged into LV and used to transduce cultures of retinal pigment epithelial cells (RPE) to investigate the role of p75 in angiogenesis (J. Zhang et al. 2014). Bertrand *et al* treated sympathetic neuron cultures with LV packaging miRNA against the receptor NRAGE, a potent activator of Jun kinase (JNK) and inducer of apoptosis which has been shown to be recruited to p75 and function as an adaptor protein between p75 and JNK. In this study LV mediated silencing of NRAGE was used to confirm observations using sympathetic neuron cultures from *NRAGE* knockout animals where they observed that NRAGE plays an important role in BDNF pro-apoptotic signalling via p75 in sympathetic neurons during development (Bertrand et al. 2009). Taken together, these studies show that LV delivery of miRNA into cells is an effective method to induce gene knockout.

LVs have also been used as a tool for *in vivo* studies: for example, Mason *et al*, injected LV into the DRG which resulted only in a handful of neurons expressing the LV encoded GFP marker, however there was evidence of transduction in non-neuronal cells suggesting that the LV was not specific enough for neuronal transduction (Mason et al. 2010). LV has been delivered *in vivo* using a range of different routes including intra-cranial injections into specific brain regions such as the hippocampus and forebrain, for example in studies investigating the role of

BDNF in the brain where among other LV vectors, LV encoding a truncated TrkB protein was bilaterally injected into rodent brains and animals were assessed for changes in memory consolidation (Heldt and Ressler 2009). LV delivery has also been used *in vivo* in combination with short hairpin RNA (shRNA) expression. For example, NGF is known to play a role in neurogenic inflammatory responses following allergen exposure and contribute to airway hyperresponsiveness (AHR). LV containing shRNA against NGF has been used to treat cultures of primary lung tissue and intratracheally to investigate inhibition of NGF as a novel therapeutic for AHR. Studies with this approach have shown that LV expressing sh-RNA against NGF attenuates lung inflammation and reduce hyperinnervation of the lung in AHR mouse models (Chen et al. 2014). Zuccaro *et al* designed shRNA against the NGF receptor p75 to be packaged into LV and injected it into the dentate gyrus of both brain hemispheres. They found p75 to be essential in governing the neuronal polarity and thus regulate the assembly of neuronal circuits in development of the adult hippocampus and cortical regions of the brain (Zuccaro et al. 2014).

3.1.3.2 Adeno-associated viral vectors

Adeno-associated viruses (AAVs) have been described as highly neurotrophic, non-pathogenic and efficient in delivering transgenes (Gompf et al. 2015; Mason et al. 2010), commonly used both *in vitro* and *in vivo*. AAVs are capable at packaging approximately 4.7-5.4 kilobases of genetic material (Gompf et al. 2015). There are nine different serotypes available which all have tropism for specific cell types. Mason *et al* investigated the different transduction profiles of AAV serotype 1-8 (AAV1-8) encoding GFP following direct injection into DRG. They found that AAV serotypes 1, 5 and 6 transduced the highest number of neurons in the DRG, (from 34-49% of DRG neurons) but overall AAV5 was found to be the most efficient with both the number of neurons transduced and the time of onset of GFP marker expression (Mason et al. 2010). AAV9 has been used to transduce primary sensory neurons as AAV9 has been shown to

transduce neurons and astrocytes. However contrary to other AAV serotypes, AAV9 is able to cross the blood brain barrier. Intrathecal injection of AAV9 has been shown to be effective in transduction of sensory neurons with over 90% of L4 DRG neurons transduced (Schuster et al. 2014).

Viral vectors have been mainly used in combination with transgenic mouse lines, for example Madison *et al* describes the use of a number of AAV vectors expressing Cre, Flpe, Flpo and Dre which target loxP, FRT (both Flpo and Flpe) and Rox sites respectively. The aim was to evaluate the use of AAV in combination with transgenic mouse lines which express reporters following Cre, Flpe, Flpo or Dre activity. Flpe was confirmed to be the least efficient, whereas Cre activity was comparable to Flpo and Dre (Madisen et al. 2015). However, there is a confound that some cell specific primers are too weak to efficiently drive transcription of a GFP or RFP marker. Therefore, Gompf *et al* described a method using a dual AAV approach using transcranial injections of an AAV encoding Cre under the control of a cell specific promoter in combination with a second AAV which encodes an inversed GFP or RFP marker protein driven by a strong neuronal or general promoter. In the presence of Cre the gene is inverted and the marker is expressed. This study found that weak cell specific promotor driving of Cre was sufficient to drive expression of Cre recombinase which then effectively drives expression of the marker protein from the second virus vector (Gompf et al. 2015). In addition to delivery of Cre recombinase, AAV9 has been used to target knockout of specific genes using short hairpin RNA (shRNA) For instance shRNA targeting TRPV1, which is known to be involved in neuropathic pain, packaged in an AAV9 vector is injected into mice to silence expression of TRPV1 in the peripheral as well as the central nervous system (Hirai et al. 2014).

3.1.4 Aim of the study

The aim of this part of the study was to specifically knockout TrkA or p75 NGF receptors using *NTRK1* fl/fl and *NGFR* fl/fl mouse lines. In this chapter, I describe the use of viral vectors to introduce Cre recombinase to DRG neurons. First, we optimised transduction of MACS purified cultures with a lenti Cre-GFP virus to knockout expression of either the TrkA receptor using DRG cultures from *NTRK1* fl/fl or p75 receptor using cultures from *NGFR* fl/fl mice *in vitro*. Second, we used intrathecal injection of adeno-associated virus serotype 9 (AAV9) to knockout expression of the TrkA or the p75 receptors in *NTRK1* fl/fl and *NGFR* fl/fl mice respectively. We characterise here the effectiveness of both methods to knockout the TrkA or p75 receptor at the protein and the RNA level. Furthermore, we show that Cre mediated knockout of TrkA or p75 result *in vitro* results in an effect on neurite outgrowth.

3.2 Methods

3.2.1 Animals

All animals were housed in a designated facility and maintained in accordance with ASPA and Home Office regulations. Animals were kept in a 12hr light dark cycle and fed *ad libitum*. All animals were sacrificed using schedule 1. TdTomato-flox-STOP-flox mice (B6;129S6-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hzel}$) originally provided by Jackson Laboratory were backcrossed to a C57Bl6 background on site (by colleague Dr Franziska Denk) and used in experiments to test if the lenti CreGFP virus was functional. All other animals used were *NTRK1* fl/fl (Ntrk1$^{tm1c(EUCOMM)Wtsi)}$ and *NGFR* fl/fl (Ngfr$^{tm1c(EUCOMM)Wtsi}$) transgenic mice obtained from the International Mouse Phenotype Consortium (IMPC) from the MRC Mammalian Genetics Unit at Harwell, both mouse strains were on a C57Bl6 background. Mice were obtained as heterozygotes and bred to homozygosity on site before being used in experiments.

3.2.2 Genotyping

Ear clips were collected from transgenic mice and digested in 450µl lysis buffer (50mM potassium chloride, 1.5mM magnesium chloride, 10mM Tris (pH 8.5), 0.45% Tergitol, 0.45% Tween-20) and 15µl of proteinase K (10mg/ml) at 55°C for 3 hours. Temperature was then increased to 95°C for 15 minutes. Following digestion samples were centrifuged at 7000rpm for 1 minute and supernatants stored at 4°C. PCR was carried out in a 20µl reaction using 18µl of a standard mix (1.5µM 5xbuffer (7.5µM $MgCl_2$) and 1.25 units GoTaq® DNA polymerase (Promega, M3175) 200nM dNTP mix (Promega, U1330) and 0.5µM oligonucleotide primers (Sigma Aldrich)) with 2ul DNA. PCR was run using the following programme: 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 58°C/60°C (*NGFR* fl/fl and *NTRK1* fl/fl respectively) for 30 seconds, 72°C for 30 seconds then finally 72°C for 7 minutes. Samples were run on a 2% agarose gel with ethidium bromide for 60 minutes at 100V and visualised under a UV lamp. For *NTRK1* fl/fl mice, WT bands were observed at 325 bp and mutant bands at 488 bp. For *NGFR* fl/fl mice, WT bands were observed at 114 bp and mutant bands at 328 bp.

Primers used were as follows:

*NTRK1*_F: GCAGTATTGCAGGTGTCCCA

*NTRK1*_R: AGGACCAAGGCTCTCACAAC

*NGFR*_F: AGACACCTCCAGTACAGAGTCAA

*NGFR*_R: CCCTTCTCCAGGTCTCATTCC

3.2.3 DRG culture

As described in the methods section of chapter 2.2.2 with the exception that B27 medium was exclusively used with or without added treatment of 10 ng/ml NGF described previously.

3.2.4 Magnetic assisted cell sorting (MACS)

As described in the methods section of chapter 2.2.3. B27 medium with or without 10 ng/ml NGF treatment was used exclusively in this chapter.

3.2.5 Lenti virus transduction *in vitro*

DRG neurons were collected and processed for non-MACS and MACS purified cultures as previously described. Following the final centrifugation at 1000 rpm for 8 min, the cell pellet was re-suspended in 100µl of plating medium and cells were counted. Based on the cell count a certain volume of virus was added to the cell suspension for each multiplicity of infection (MOI) tested; since the MOI defines how many viral particles per neuron are present, 30 viral particles per neuron are added for MOI 30. We used a CMV-Cre-GFP lentivirus at a titre of 1x10^8 CFU/ml (Cellomics Technology, PLV-10039). Cells were incubated with the virus for 20 min at 37^{o}C before they were plated onto PLL and matrigel coated coverslips as previously described for DRG and MACS purified DRG cultures. For RNA extraction, 4-well plates were coated directly (no coverslips used) with PLL and matrigel following the same protocol as for coverslips. Cultures were grown in B27 medium in the absence of NGF unless otherwise stated. Medium was changed after 24 hours and every subsequent 48 hours until fixation and staining.

3.2.6 Immunocytochemistry

As described in the methods section of chapter 2.2.5.

The following primary antibodies were used for this chapter: 1:1000 anti-GFP chicken polyclonal (Abcam, ab13970) and 1:1000 anti-β-III tubulin mAb (Promega, G712A) except for AAV Cre-eGFP neurite outgrowth experiments where 1:1000 anti-β-III tubulin rabbit polyclonal (Abcam, ab18207) was used.

The following secondary antibodies were used for this chapter: 1:1000 goat anti-chicken IgY (H+L) Alexa Fluor® 488 (ThermoFisher, A11039), 1:1000 donkey anti-mouse IgG (H+L) Alexa Fluor® 546 (ThermoFisher, A10036) and 1:1000 donkey anti-rabbit IgG (H+L) Alexa Fluor® 568 (ThermoFisher, A10042).

3.2.7 AAV9 virus injection

Intrathecal (IT) administration of AAV9 Cre-eGFP virus was optimised and performed by another PhD student in the lab, Nikita Khovanov. Briefly, mice were anaesthetised with isofluorane and given 50µl of 0.5% analgesic carprive and 100µl of saline subcutaneously. A 3-4 cm long area from the base of the neck was shaved. An incision of 1.5 cm length at the T13 vertebrae was made and the muscle removed to expose the membrane between the vertebrae T12 and T13. A gauge 30 needle was then used to open the dura. A narrow cannula (diameter 0.02cm) preloaded with 5µl of AAV9 Cre-eGFP was inserted into the intrathecal space down the left hand side of the dorsal vein. Virus was administered at a rate of 1.2 µl/min, then left for 2 min to equilibrate before the cannula was removed. The skin was closed using wound clips and animals were left to recover. After 10 days wound clips were removed. 14 days post-surgery animals were sacrificed using schedule 1 and tissue collected fresh, without perfusion.

3.2.8 Immunohistochemistry

Mice were sacrificed and fresh tissue was collected. Both L4 DRGs were collected and fixed in 2% PFA for 2 hours at 4^{o}C before being washed three times for 20 mins in 1xPBS at 4^{o}C. Tissue was then incubated in 30% sucrose for 24 hours at 4^{o}C before being embedded in optimal cutting temperature (OCT) compound and rapidly frozen on a bed of dry ice and isopentane. Tissue was stored at -80^{o}C before cutting on a cryostat. DRG sections were cut at 10µm thickness, collected on slides and left to dry overnight and stored at -20^{o}C prior to staining.

The following primary antibodies were used: 1:1000 anti-GFP chicken polyclonal (Abcam, ab13970), 1:1000 anti-β-III tubulin rabbit polyclonal (Abcam, ab18207), 1:500 anti-p75 NGF receptor rabbit polyclonal (Abcam, ab8875) and 1:200 anti-TrkA goat polyclonal (R&D systems, AF1056).

The following secondary antibodies were used: 1:1000 goat anti-chicken IgY (H+L) Alexa Fluor® 488 (ThermoFisher, A11039), 1:1000 donkey anti-rabbit IgG (H+L) Alexa Fluor® 568 (ThermoFisher, A10042) and 1:1000 chicken anti-goat IgG (H+L) Alexa Fluor® 488 (ThermoFisher, A21467).

Staining was performed as follows. Firstly, sections were left for 30 min at RT to equilibrate and the edges of the slide marked with a PAP pen to form a hydrophobic barrier. Sections were blocked in 10% normal donkey serum (NDS) (Abcam, ab1666643) in 0.2% PBS-Triton X-100 (0.2% triton-100 and 0.1% sodium azide in 1xPBS) for 1 hour at RT. Blocking solution was then removed and replaced with primary antibody solution (antibody diluted in 0.2% PBS-Triton X-100) and sections were left overnight at RT in a humidified chamber. Sections were then washed four times for 5 min in 1xPBS before a solution of secondary antibody (secondary antibody diluted in 0.2% PBS-Triton X-100) was added. Slides were incubated the secondary antibody solution for 2 hours at RT in a dark humidified chamber before being washed four times for 5min in 1xPBS. Sections were then mounted onto microscope slides using Fluoromount G with DAPI (eBioscience, 00-4959-52).

3.2.9 RNA isolation and cDNA amplification post *in vitro* lentivirus treatment

Following MACS purification control samples were collected directly by centrifugation (7000 rpm for 6 mins). Cells were re-suspended in 500 µl RLT buffer with 5 of µl β-mercaptoethanol, vortexed for 30 seconds and then stored at -80^{o}C. The RNA of samples treated with CMV-Cre-GFP lenti virus and cultured for 1-3 days was isolated as follows. The medium was aspirated

and 500 µl of RLT buffer with 5 µl of β-mercaptoethanol was added directly to the well. Cells were scraped off and collected prior to vortexing and storing as above. Once all samples were collected, RNA was isolated using the Qiagen RNeasy Micro kit according to the manufacturer's protocol from step 4 onwards (Qiagen, 74004). RNA quantity and quality was measured with the Agilent RNA 6000 pico kit for the bioanalyser (Agilent technologies, 5067-1535) using an Agilent2100 Bioanalyzer (Agilent, Santa Clara, CA).

RNA was then reverse transcribed to cDNA and amplified using the QuantiTect® Whole Transcriptome kit (Qiagen, 207045). 150pg of RNA per sample was used to produce cDNA of a final concentration of 3 ng/µl following the manufacturer's protocol.

3.2.10 RNA isolation and cDNA amplification post IT AAV9 Cre-eGFP treatment

Both L3 DRGs of one animal were collected and pooled in RNAse free tubes, snap frozen in liquid nitrogen and stored at -80^{o}C. Prior to RNA isolation, tubes were kept on dry ice or in liquid nitrogen. DRGs were transferred to RNAse free 2ml flat bottomed tubes with 500 µl Qiazol (Qiagen, 79306) and tissue was homogenised using a hand held homogeniser in two 10 second pulses. Once all the samples were homogenised, all of the samples were checked for un-homogenised tissue aggregates and re-homogenised if necessary. Samples were incubated for 5 mins at RT, meanwhile phase lock gel (PLG) columns were pre-spun at 12000 rpm for 2 mins. Homogenates were added to the PLG columns and 100 µl of chloroform was added and the tubes were shaken for 15 seconds. Tubes were then incubated at RT for 3 mins followed by 15 mins centrifugation at 12000 rpm at 4^{o}C. The aqueous phase was then poured off into a fresh RNAse free tube and 1.5 volume of 70% Ethanol in DEPC (made fresh) was added and tubes inverted to mix. The solution was then transferred to RNAeasy spin columns 700 µl at a time and centrifuged for 1 min at 11000 rpm and the flow through discarded. This process was repeated until all of the sample had been added to the column. From here the RNA was

isolated using reagents from the RNAeasy micro kit (Qiagen, 74004) and following the manufacturer's protocol from step 6 onwards, before the quantity and quality of RNA was measured using the Agilent RNA 6000 pico kit for the bioanalyser (Agilent technologies, 5067-1535) using an Agilent2100 Bioanalyzer (Agilent, Santa Clara, CA).

For reverse transcription, RNA was diluted in DEPC water to a final concentration of 250 ng/µl in a final volume of 12 µl. 1 µl of 50 mM random primers (Promega, C1181) and 1 µl of 10 mM dNTPs (Promega, U1330) were added to each reaction and placed in a thermocycler at 65^{o}C for 5 mins. Samples were then cooled to 4^{o}C for 1 min before 6 µl of a second mastermix was added for a final reaction volume of 20 µl (4 µl 5x first strand buffer, 1 µl 0.1 M DTT and 1 µl 200 U/µl Superscript III RT (Life technologies, 18080-044)). Samples were placed in a thermocycler and the following programme was run: 25^{o}C for 5 min, 50^{o}C for 60 min, 70^{o}C for 15 min, 4^{o}C until the programme was stopped at which point cDNA was stored at -20^{o}C.

3.2.11 RT-PCR

3.2.11.1 Optimisation of RT-PCR primers for *NTRK1* and *NGFR*

Primers for *NTRK1* and *NGFR* genes were designed using the Primer blast tool of Pubmed, purchased from Sigma Aldrich and then tested for efficiency against a standard tissue sample for efficiency. Primers were designed to target the appropriate gene at sites before, over, after and further downstream (end) from the loxP floxed sites. Briefly, tissue for a standard tissue sample was collected from a C57Bl WT mouse including small pieces of tissue (≤ 1 mm^2) from heart, lung, brain, liver, kidney, bladder, muscle, DRG, spinal cord and sciatic nerve and snap frozen in liquid nitrogen. Tissue was dropped into a fresh RNAse 2ml flat bottomed tube containing 500 µl of Qiazol and homogenised using a hand held homogeniser in two 10 second pulses and re-homogenised if necessary. The samples were incubated for 5 mins at RT, meanwhile phase lock gel (PLG) columns were pre-spun at 12000 rpm for 2 mins.

Homogenates were added to the top of the PLG columns and 100 µl of chloroform was added. The tubes were shaken for 15 seconds and then left to incubate at RT for 3 mins. Tubes were then centrifuged for 15 mins at 12000 rpm at 4^{o}C. The aqueous phase was then poured off into a fresh RNAse free tube and 1.5 volume of 100% Ethanol in DEPC (made fresh) was added to the tubes and inverted to mix. The solution was then transferred to RNAeasy spin columns 700 µl at a time and centrifuged for 1 min at 11000 rpm and the flow through discarded. This process was repeated until all of the sample has been added to the column. From here the RNA was then isolated using reagents from the RNAeasy mini kit (Qiagen, 217004) and following the manufacturer's protocol from step 11 onwards, before the quantity and quality of RNA was measured using the nanodrop.

For reverse transcription of RNA to cDNA from tissue samples RNA was first diluted in DEPC water to a final concentration of 2.5 µg/µl in a final volume of 12 µl. 1 µl of 50 mM random primers (Promega, C1181) and 1 µl of 10 mM dNTPs (Promega, U1330) were added to each reaction and tubes were placed in a thermocycler at 65^{o}C for 5 mins. Samples were then cooled to 4^{o}C for 1 min before 6 µl of a second mastermix (4 µl 5x first strand buffer, 1 µl 0.1 M DTT and 1 µl 200 U/µl Superscript III RT (Life technologies, 18080-044)) was added for a final reaction volume of 20 µl. Samples were placed in a thermocycler and the following programme run: 25^{o}C for 5 min, 50^{o}C for 60 min, 70^{o}C for 15 min then cool to 4^{o}C, before cDNA was then stored at -20^{o}C. To carry out the RT-PCR cDNA was diluted to 60 ng/µl, and then serially diluted to 30, 10, 3, 1, 0,3 and 0.1 ng/µl to assess the primer efficiency. For all reactions, a melt curve was calculated and the RT-PCR products were run on a 2% agarose gel to verify that the product size matched the predicted length and there was no non-specific binding and amplification.

A summary of all tested primers for *NTRK1* and *NGFR* can be found in **Supplementary tables 3.1 and 3.2**.

3.2.11.2 RT-PCR reaction

Firstly, a mastermix for each primer pair was prepared, consisting of 5 µl of 2x Lightcycler[480] SYBR green I master (Roche, 04887352001), 3.5 µl of RNAse free water and 0.5 µl of 10 µM forward and reverse primer mix (Sigma Aldrich) per reaction. 9 µl of mastermix was then pipetted into a 384 well plate and 1 µl of cDNA was added to each well. The plate was then sealed and centrifuged in a plate spinner for 20 seconds before being placed into a Roche Lightcycler II RT-PCR machine. Plates were all run using the following programme: pre-incubation at 95^{o}C for 5 mins, then 45 cycles of amplification at 95^{o}C, 60^{o}C and 72^{o}C for 10 seconds each with a single acquisition after each 72^{o}C incubation. A melt curve programme was run immediately after: 95^{o}C for 5 sec before cooling to 65^{o}C for 1 min and the temperature ramped up to 97^{o}C with continuous acquisition. Samples were then cooled to 4^{o}C.

Primers used in this chapter were as follows:

*GAPDH*_F: ATGGTGAAGGTCGGTGTGA

*GAPDH*_R: AATCTCCACTTTGCCACTGC

*NTRK1*_F: GAAGAATGTGACGTGCTGGG

*NTRK1*_R: GAAGGAGACGCTGACTTGGA

*NGFR*_F: CCGCTGACAACCTCATTCC

*NGFR*_R: GGCTGTTGGCTCCTTGTTTATTT

3.2.12 Imaging and analysis

Mosaic images were acquired at 200x using Carl Zeiss microscope and Axiovision software and neurite outgrowth assays were performed as described in the methods section of chapter 2.2.7.

For lenti viral CreGFP treatment, the number of cells remaining in culture post fixing was calculated using the cell counter plugin of Fiji. The percentage of neurons successfully transduced was measured as the percentage of neurons expressing GFP above control cells. This was calculated by first circling all cells imaged in Fiji and then measuring the amount of Fluorescence (measured as IntDen) in Fiji. For each of the control conditions the fluorescence intensity for all cells was measured and an average and standard deviation of fluorescence was calculated. The average was then added to two times the standard deviation and a 95% confidence interval (CI) for fluorescence was calculated, thus a baseline fluorescence accounting for 95% of control cells was set. Neurons in culture treated with lenti virus expressing GFP above the respective 95% control threshold were said to be GFP positive and therefore successfully transduced with lenti virus and the percentage of GFP expressing neurons was calculated.

Percentages of GFP, TrkA and p75 positive neurons post IT AAV9 Cre-eGFP was calculated by counting the number of cells expressing the marker of interest as a percentage of the total number of cells in the DRG section using the cell counter plugin of Fiji across a minimum of 3 sections of both L4 DRG for each animal.

Fold change from RT-PCR was calculated using the delta-delta Ct (Cq) normalised to the expression of the *GAPDH* house-keeping gene.

3.2.13 Statistics

Graphs were plotted and statistical tests performed using GraphPad Prism 7 software.

One-way ANOVA with Tukey multiple comparison test was performed for all analysis except for RT-PCR expression of NTRK1 and NGFR following lenti virus Cre-GFP treatment where a two-way ANOVA with Tukey multiple comparison test was performed. Linear regression analysis was also performed to compare expression of GFP with TrkA or p75 expression in *NTRK1* fl/fl and *NGFR* fl/fl mice treated with AAV9 Cre-eGFP virus.

3.3 Results

3.3.1 Validation of lenti virus as a tool for knockout of floxed genes

Assessment of the transgenic mouse line will be reported in chapter 4, but we observed no alterations in DRG development. In this chapter, we investigate viral transduction of DRG, beginning with lenti virus. To investigate the effect of knock out of floxed genes *in vitro* we first optimised a method for transducing MACS purified cells using lenti viral treatment. DRG cultures without MACS purification from tdTomoto-flox-STOP-flox mice were treated with various multiplicity of infection (MOI) of a lenti virus encoding a Cre-GFP fusion protein (lenti Cre-GFP) and expression of tdTomato and GFP without antibody amplification was observed with all concentrations of viral particles showing effective transduction and the Cre recombinase to be functional (**Fig 3.2a**). For the treatment of MACS purified cells with lenti Cre-GFP virus different MOI and number of incubation days *in vitro* were used for optimisation and the percentage of cells expressing GFP were measured. The baseline level was set as the 95% CI for the corresponding basal fluorescence for the MOI 0 control, and using this baseline the percentage of cells treated with lenti virus expressing GFP above this threshold was

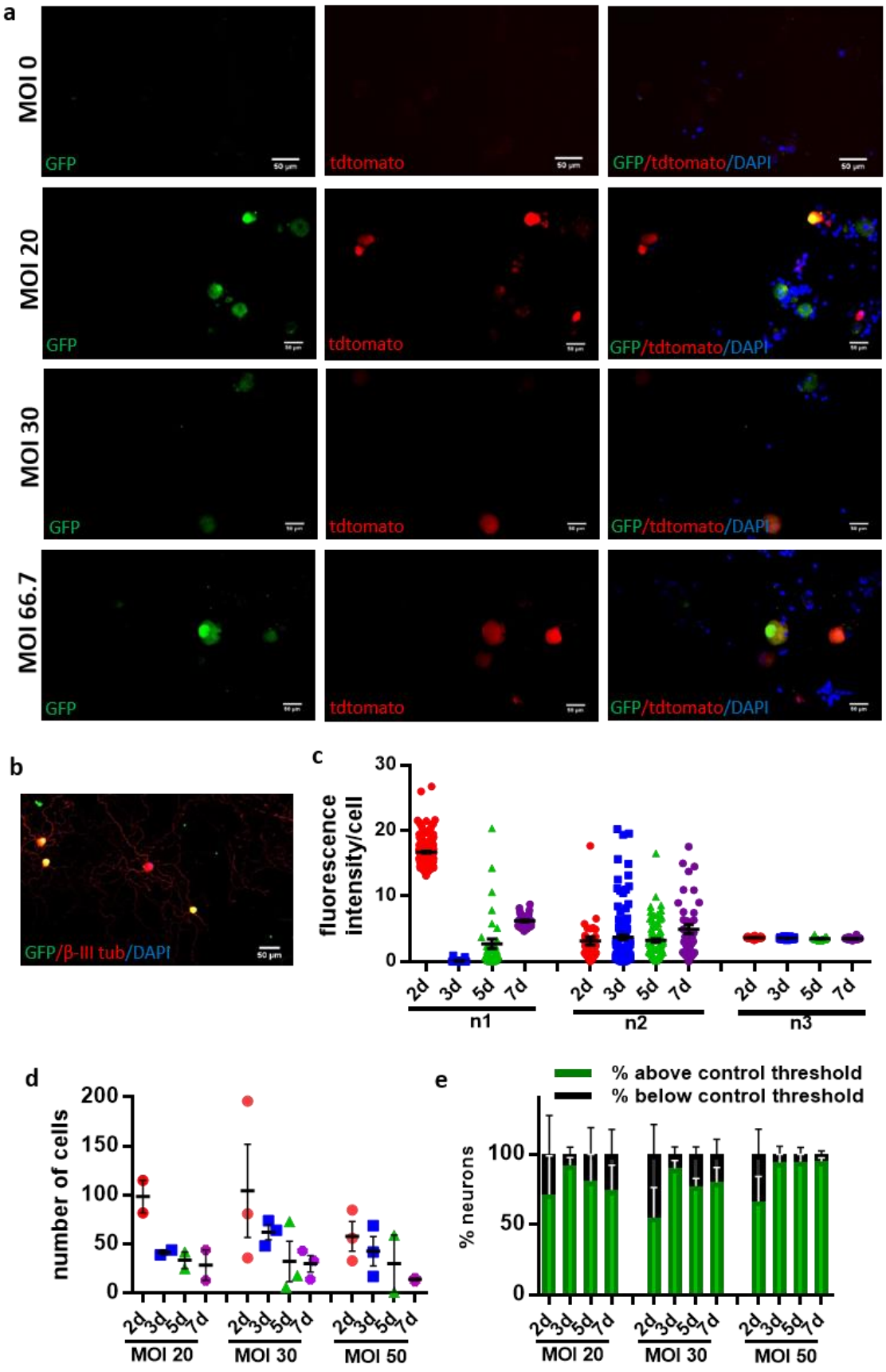

a
MOI 0
MOI 20
MOI 30
MOI 66.7
GFP
tdtomato
GFP/tdtomato/DAPI
b
GFP/β-III tub/DAPI
c
fluorescence intensity/cell
30
20
10
0
2d 3d 5d 7d
n1
n2
n3
d
number of cells
200
150
100
50
0
MOI 20
MOI 30
MOI 50
e
% above control threshold
% below control threshold
% neurons
100
50
0

***Figure 3.2 Optimisation of lenti CreGFP transduction of MACS purified DRG neurons**.*

***a-** Representative immunocytochemistry of non-MACS DRG cultures from tdTomato flox-STOP-flox mice treated with various MOI of lenti CreGFP. **b-** Representative ICC of optimal lenti CreGFP in MACS purified DRG culture, MOI 30 cultured for 3 days. **c-** Control fluorescence from untreated cells used to calculate 95% CI of the fluorescence intensity/cell after 2, 3, 5 and 7 days in culture (2d, 3d, 5d and 7d respectively) from each of the individual cultures (n). **d-** Number of cells surviving following treatment with varying MOI of lenti virus. **e-** Percentage of MACS purified neurons treated with varying MOI expressing GFP above the 95% CI threshold set from the control GFP expression. All n=2-3 independent cultures.*

measured. 64.3% ± 8.4% of cells expressed GFP after 2 days, 92.3% ± 2% after 3 days, 84.4% ± 9.2% after 5 days and 83.2% ± 10.6% after 7 days (**Fig 3.2b, c and e**). The number of surviving cells was measured by counting the remaining cells in culture. It was found that there were fewer cells 5 and 7 days (average 28 ± 7 cells) *in vitro* compared to 2 and 3 days (average 68 ± 27 cells). This correlated with a larger amount of cell debris observed after 5 and 7 days in culture (**Fig 3.2d**). Taken together we determined the optimal conditions for lenti Cre-GFP treatment were an MOI 30 for 3 days *in vitro* (**Fig 3.2b**).

3.3.2 Lenti CreGFP treatment of *NTRK1* fl/fl and *NGFR* fl/fl animals was not optimal for observing changes in neurite outgrowth

To investigate potential differences in neurite outgrowth following lenti Cre-GFP mediated knockout of either TrkA using *NTRK1* fl/fl mice or p75 using *NGFR* fl/fl mice respectively, DRGs were isolated and MACS purified from both strains. Cultures were treated with lenti Cre-GFP for 3 days *in vitro*. The purified cells were subsequently treated with NGF (either at the time of

plating or after 1 or 2 days in culture, d0, d1 and d2 respectively) to investigate the effect of NGF on neurite outgrowth in either NGF receptor expressing or NGF receptor knock out cells.

First we investigated the effect of NGF on neurite outgrowth following lenti Cre-GFP mediated knock out of TrkA in MACS purified cultures from *NTRK1* fl/fl animals. In cultures from *NTRK1* fl/fl animals there was significant increase in neurite outgrowth between MOI 0 and 30 lenti CreGFP when treated with NGF after 2 days *in vitro* from 2658 ± 247 µm in control to 3840 ± 82 µm in MOI 30 treated neurons (p= 0.0421). There was also a significant increase in neurite outgrowth between MACS purified neurons treated with a MOI 30 lenti CreGFP and NGF at the time of plating compared to treatment with NGF after 1 (p=0.0480) and 2 (p=0.0384) days *in vitro*, one-way ANOVA with Tukey post-hoc. NGF treatment at the time of plating and treatment of neurons with lenti CreGFP virus at MOI 30 resulted in overall neurite outgrowth of 2633 ± 386 µm, compared to 4038 µm when NGF treatment was added after 1 day *in vitro* and 3840 ± 32 µm when NGF was added after 2 days *in vitro* (**Fig 3.3a and b**). There were no significant differences between any other neurite outgrowth measures. In controls cultures treated with an MOI 0 there was average neurite outgrowth of 2679 ± 412 µm in the absence of NGF, 2824 ± 64 µm following NGF treatment at the time of plating, 3414 µm with NGF treatment after 1 day *in vitro* and finally neurons treated with MOI 30 in the absence of NGF showed an average neurite outgrowth of 3443 ± 150 µm. Additional neurite outgrowth measurements longest neurite (p=0.0172) and mean neurite length (p=0.0360) both showed a significant increase in outgrowth between MOI 0 and 30 treated MACS purified cultures treated with NGF after 2 days *in vitro.* However, other additional neurite outgrowth measurements like shortest neurite, mean neurite length, number of neurites per neurons, total length from longest branch and overall neurite outgrowth normalised to number of neurites all showed no significant differences following treatment with lenti CreGFP or NGF, one-way ANOVA with Tukey post-hoc (**Supplementary Fig 3.1**).

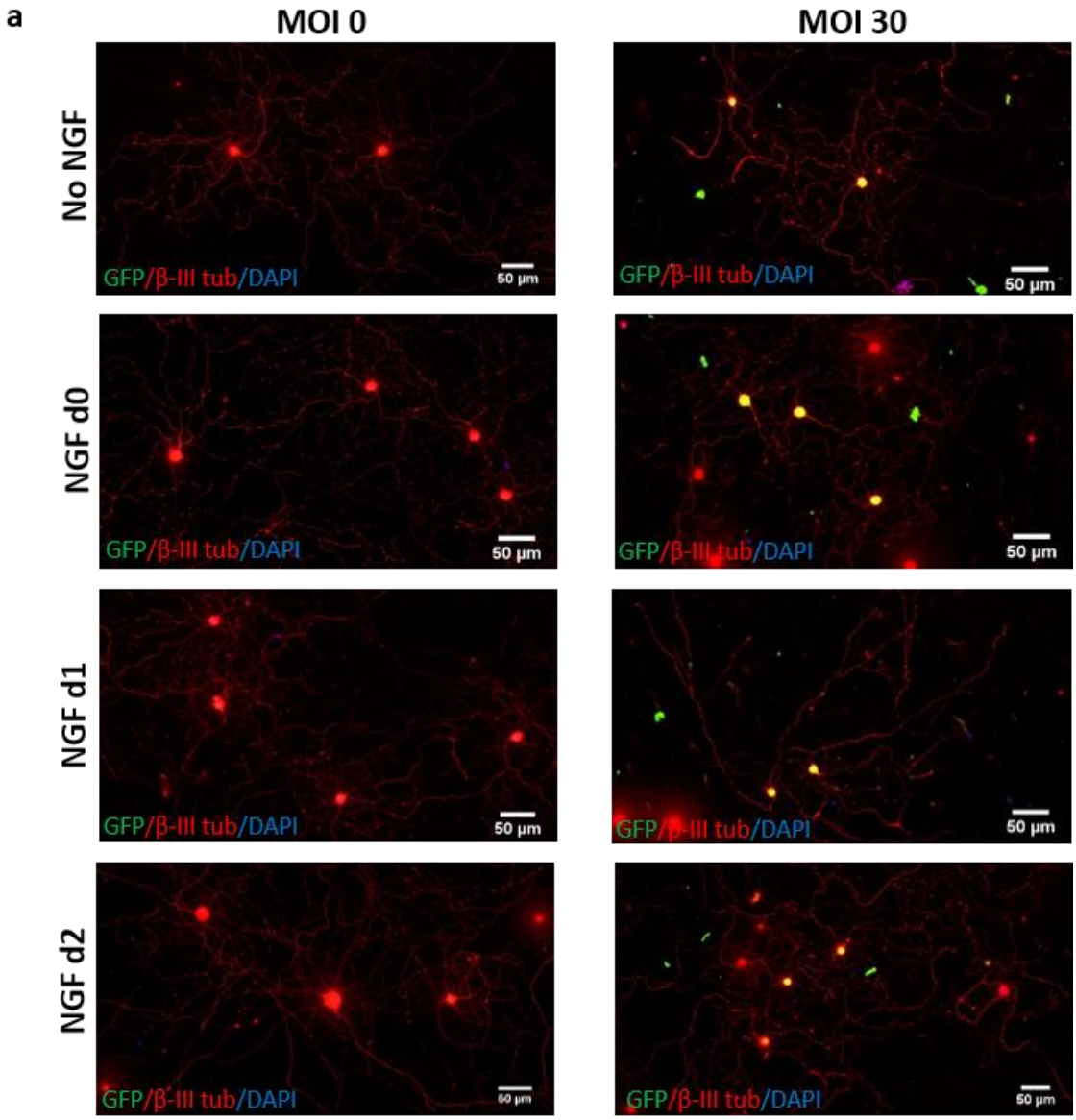

a
MOI 0
MOI 30
No NGF
NGF d0
NGF d1
NGF d2
GFP/β-III tub/DAPI
50 μm
b
Total neurite outgrowth (μm)
4500
4000
3500
3000
2500
2000
*
*
*
No NGF
NGF d0
NGF d1
NGF d2
No NGF
NGF d0
NGF d1
NGF d2
MOI 0
MOI 30

Figure 3.3 Lenti CreGFP mediated KO of TrkA in MACS purified DRG from NTRK1 fl/fl mice does not reduce neuronal response to NGF*. **a-** Representative immunocytochemistry of NTRK1 fl/fl MACS purified cultures treated with NGF at 0, 1 or 2 days after treatment with lenti CreGFP at MOI 0 or 30. **b-** Quantification of neurite outgrowth 3 days post treatment with lenti CreGFP and NGF treatment at varying time points after virus treatment (MOI 0 NGF d2 vs MOI 30 NGF d2 p=0.0421, MOI 30 NGF d0 vs MOI 30 NGF d1 p=0.0480, MOI 30 NGF d0 vs MOI 30 NGF d2 p=0.0384). One-way ANOVA, Tukey post hoc, n=2 individual cultures.*

MACS purified neurons from DRGs isolated from *NGFR* fl/fl animals and treated with lenti Cre-GFP and NGF however showed no significant differences in total neurite outgrowth *in vitro.* For control neurons treated with MOI 0 the average neurite outgrowth in the absence of NGF was 2625 ± 1072 µm, following NGF treatment at the time of plating 2770 ± 204 µm, following NGF treatment after 1 day *in vitro* 2510 ± 480 µm and 2 days *in vitro* 2967 µm. For MACS purified neurons treated with lenti Cre-GFP at a MOI 30 the average neurite outgrowth in the absence of NGF was 2508 ± 389 µm, following NGF treatment at the time of plating 3114 ± 162 µm, following NGF treatment after 1 day *in vitro* 2836 ± 637 µm and 2 days *in vitro* 3468 µm (**Fig 3.4a and b**). Similarly, additional neurite outgrowth measurements longest, shortest, mean, number of neurites, total length from longest neurite branch and overall neurite outgrowth normalised to the number of neurites all showed no significant differences following lenti Cre-GFP mediated knockout of p75 in NGFR fl/fl animals, one-way ANOVA with Tukey post-hoc (**Supplementary Fig 3.2**).

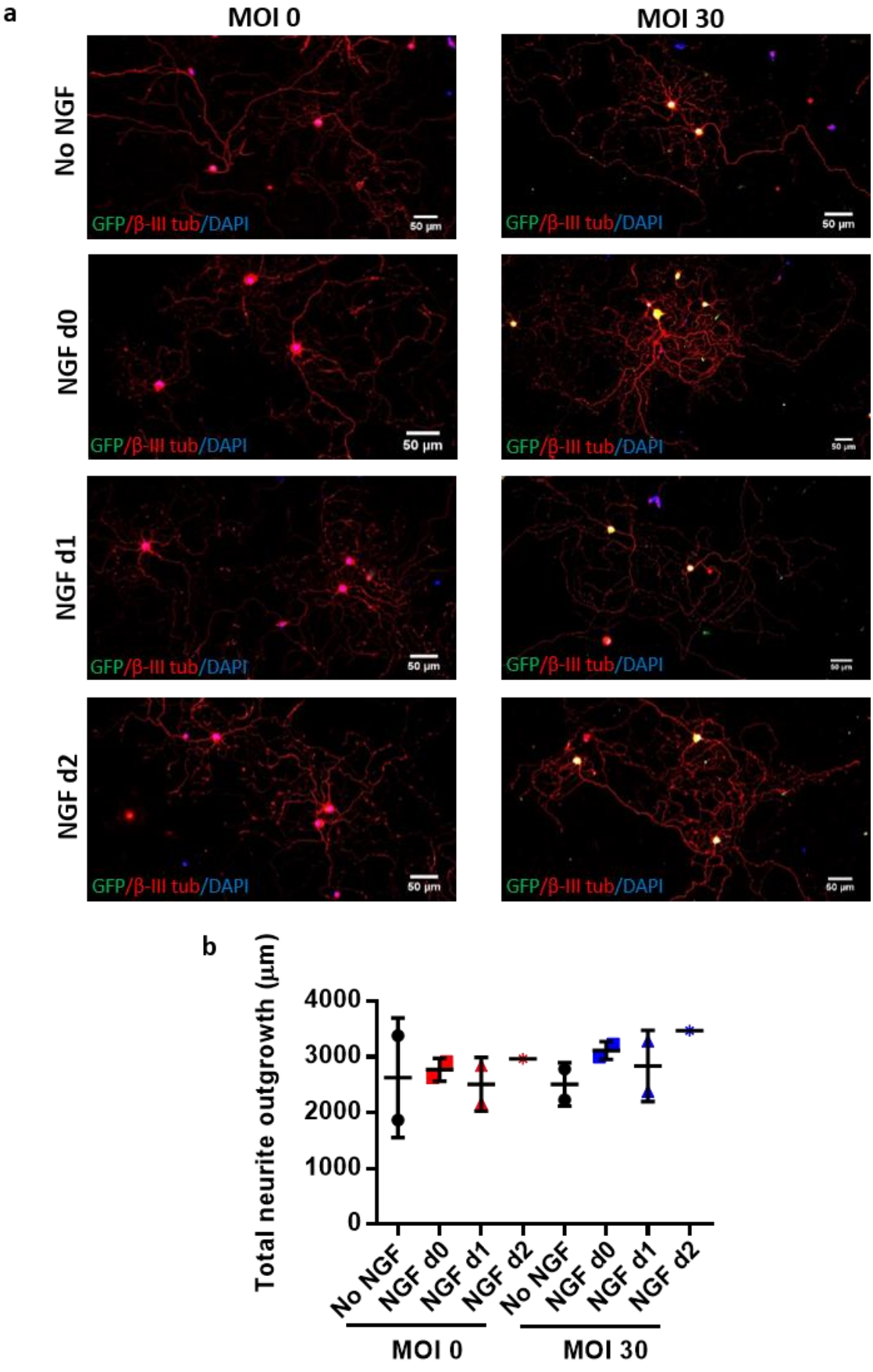

a
MOI 0
MOI 30
No NGF
NGF d0
NGF d1
NGF d2
GFP/β-III tub/DAPI
50 µm
b
Total neurite outgrowth (µm)
4000
3000
2000
1000
0
No NGF
NGF d0
NGF d1
NGF d2
No NGF
NGF d0
NGF d1
NGF d2
MOI 0
MOI 30

Figure 3.4 Lenti CreGFP mediated KO of p75 in MACS purified DRG from NGFR fl/fl mice does not affect neuronal response to NGF. a- *Representative ICC of NGFR fl/fl MACS purified cultures treated with NGF at 0, 1 or 2 days after treatment with lenti CreGFP at MOI 30.* ***b-*** *Quantification of neurite outgrowth 3 days post treatment with lenti CreGFP and NGF treatment at varying time points after virus treatment. None of the differences in neurite outgrowth were statistically significant. One-way ANOVA, Tukey post hoc, n=2 individual cultures.*

Next, we investigated the effectiveness of knockout of the *NTRK1* and *NGFR* genes following treatment of MACS purified neurons with lenti Cre-GFP using RT-PCR. Investigations of neurite outgrowth following TrkA or p75 knockout from DRGs from *NTRK1* fl/fl and *NGFR* fl/fl mice respectively showed no significant differences in neurite outgrowth. We hypothesised that this could be the result of insufficient gene knockdown or that we are observing a ceiling effect on outgrowth. To further investigate this, we analysed the gene expression for TrkA or p75 by RT-PCR on a small numbers of MACS purified neurons from *NTRK1* fl/fl and *NGFR* fl/fl mice treated with lenti CreGFP for up to 3 days *in vitro* in the absence of NGF. We found that compared to control expression levels of untreated neurons isolated straight from the MACS column there was no significant change in expression of either the *NTRK1* or the *NGFR* gene from purified neurons treated with lenti Cre-GFP over 1-3 days from cultures from both *NTRK1* fl/fl (**Supplementary Fig 3.3a**) or *NGFR* fl/fl (**Supplementary Fig 3.3b**) mice, two-way ANOVA with Tukey post-hoc. This could suggest that there is indeed insufficient knockout of *NTRK1* or *NGFR* following lenti Cre-GFP treatment. However, this could also be the result of degradation or a low concentration of RNA collected from MACS purified neurons. On the other hand, we found the quality of the RNA isolated to be good and the quantity of the RNA was found to be

suitable for use with a cDNA amplification kit suggesting this is not the case (**Supplementary Fig 3.3c and d**).

3.3.3 Intrathecal injection of AAV9 into *NTRK1* fl/fl or *NGFR* fl/fl mice effectively knocks down expression of TrkA or p75 at the RNA and protein level

Since knockout of TrkA and p75 using lenti CreGFP viral treatment *in vitro* was found not to be efficient we next used an adeno-associated virus (AAV) to achieve *in vivo* knockout of TrkA or p75. We used an AAV serotype 9, which has previously been shown in our group to effectively transduce DRG neurons when injected via a cannula intrathecally (IT). For example, we previously found that IT injection of AAV9 encoding eGFP protein effectively transduces 67.2% ± 16.5% of lumbar DRG neurons, 13.7% ± 6.6% of thoracic DRG neurons and 60.6% ± 6.2% cervical DRG neurons (unpublished data from a PhD student in the lab, Nikita Khovanov).

We injected AAV9 Cre-eGFP into *NTRK1* fl/fl animals IT and quantified the percentage of GFP positive transduced neurons 2 and 4 weeks post AAV9 Cre-eGFP. We found 52.9% ± 10.3% of the DRG neurons to be GFP positive 2 weeks and 30.0% ± 15.1% GFP positive 4 weeks post AAV9 Cre-eGFP injections. This data is pooled from both L4 DRGs. Interestingly, there was a significant decrease in the number of GFP positive neurons from 2 to 4 weeks post injection, p=0.0372 one-way ANOVA with Tukey post-hoc **(Fig 3.5a and b**). Next, we stained the DRGs against TrkA and quantified its expression. This revealed that there was a significant decrease in TrkA expression from 38.5% ± 6.5% in un-injected WT DRGs to 15.0% ± 4.9% at 2 (p=0.0017) and 19.8% ± 6.8% at 4 (p=0.0092) weeks post AAV9 Cre-eGFP injection. This data is pooled from both L4 DRGs, one-way ANOVA with Tukey post-hoc (**Fig 3.5a and c**). Furthermore, we investigated if there was a difference in transduction between the left and right L4 DRG. Indeed, we detected in almost all (91%) injected animals there was a varying degree of

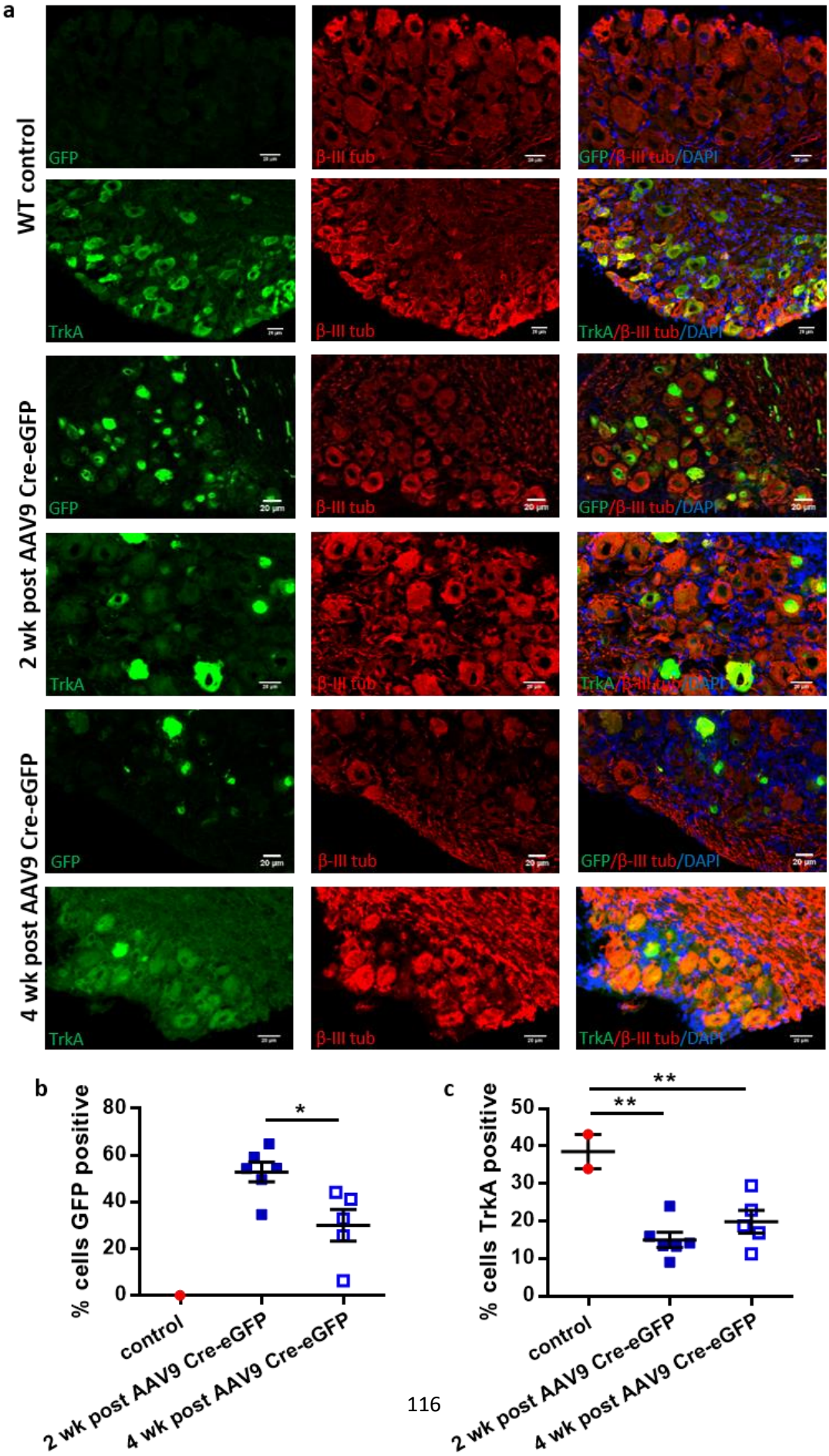

a
WT control
2 wk post AAV9 Cre-eGFP
4 wk post AAV9 Cre-eGFP
GFP
β-III tub
GFP/β-III tub/DAPI
TrkA
TrkA/β-III tub/DAPI
b
% cells GFP positive
control
2 wk post AAV9 Cre-eGFP
4 wk post AAV9 Cre-eGFP
c
% cells TrkA positive

Figure 3.5 Intrathecal injection of AAV9 Cre-eGFP into NTRK1 fl/fl animals reduces the protein expression of TrkA in the L4 DRG. a- *Representative immunocytochemistry of GFP and TrkA expression in L4 DRG 2 and 4 weeks post AAV9 Cre-eGFP administration. WT controls tissue used was taken from C57Bl wildtype mice not treated with AAV9 Cre-eGFP.* ***b-*** *Quantification of GFP (p=0.0372) and* ***c*** *TrkA (control vs 2 wk AAV9 Cre-eGFP p=0.0017, 4wk AAV9 Cre-eGFP p=0.0092) expression in L4 DRG following intrathecal AAV9 Cre-eGFP administration in NTRK1 fl/fl mice. One-way ANOVA with Tukey post-hoc, n=2-6 animals.*

unilateral transduction as assessed by GFP and TrkA expression (**Supplementary Fig 3.4a and c**).

We then performed the same IT injections in *NGFR* fl/fl animals and observed again an increase in the expression of GFP to 39.0% ± 23.0 % and 16.3% ± 8.9% at 2 and 4 weeks post AAV9 Cre-eGFP injection respectively, pooled data from both L4 DRGs (**Fig 3.6a and b**). After staining for p75 we found a trend towards a reduction in the expression of p75 from 38.5% ± 6.5% in control DRG to 27.1% ± 15.3% at 2 and 18.5% ± 11.8% at 4 weeks post AAV9 Cre-eGFP injection, however this data was not significant. Data pooled from both L4 DRGs, one-way ANOVA with Tukey post-hoc (**Fig 3.6a and c**). As with the other animals, AAV9 Cre-eGFP injection into *NGFR* fl/fl mice also show a degree of unilateral transduction of virus between left and right L4 DRG as seen by GFP and p75 expression, but only in approximately 30% of mice injected (**Supplementary Fig 3.4b and d**).

Correlation of GFP and TrkA or p75 expression in L4 DRG of *NTKR1* fl/fl (R^2= 0.1987, 95% CI -0.3126 to -0.0102) or *NGFR* fl/fl (R^2= 0.4284, 95% CI -0.6658 to -0.1813) mice 2 and 4 weeks post AAV9 Cre-eGFP injection showed a negative correlation between GFP and TrkA or p75 expression respectively. However, this trend was not significant (**Fig 3.7a**). Despite the

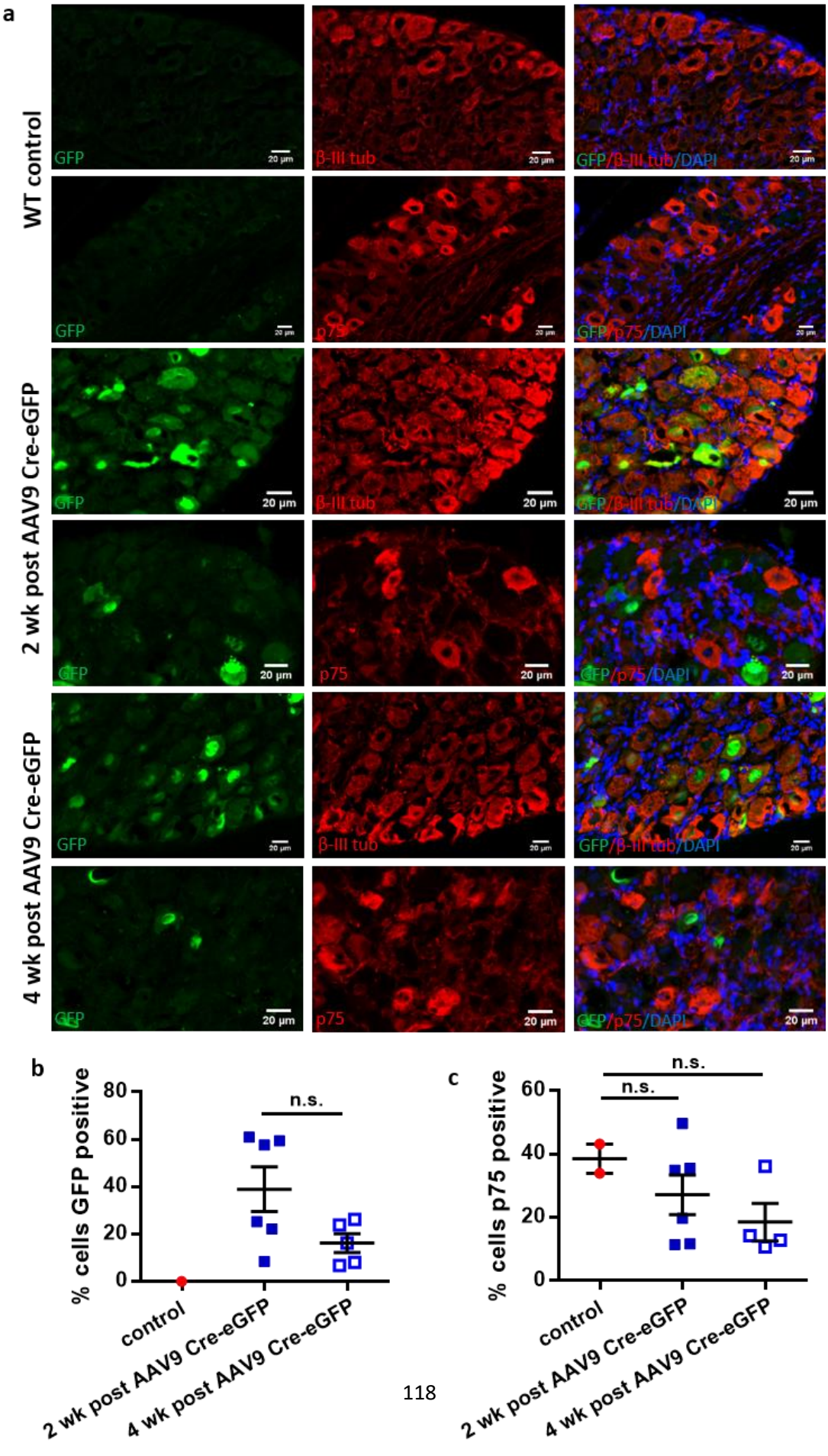

a
WT control
2 wk post AAV9 Cre-eGFP
4 wk post AAV9 Cre-eGFP
GFP
β-III tub
GFP/β-III tub/DAPI
p75
GFP/p75/DAPI
20 μm
b
% cells GFP positive
n.s.
control
2 wk post AAV9 Cre-eGFP
4 wk post AAV9 Cre-eGFP
c
% cells p75 positive
n.s.
n.s.

Figure 3.6 Intrathecal injection of AAV9 Cre-eGFP into NGFR fl/fl animals trends towards a reduction in the protein expression of p75 in the L4 DRG. a- *Representative immunocytochemistry of GFP and p75 expression in L4 DRG 2 and 4 weeks post AAV9 Cre-eGFP administration. Control tissue used here was taken from C57Bl mice not treated with AAV9 Cre-eGFP.* ***b-*** *Quantification of GFP and* ***c*** *p75 expression in L4 DRG following intrathecal AAV9 Cre-eGFP administration in NGFR fl/fl mice. One-way ANOVA with Tukey post-hoc, all tests not significant, n=2-6 animals.*

observed laterality of GFP and TrkA/p75 expression between the left and right L4 DRG the trend for negative correlation between GFP and TrkA or p75 expression was maintained in both DRGs of *NTRK1* fl/fl (left R^2 = 0.1355, 95% CI -0.4343 to 0.1352 and right R^2 =0.5018, 95% CI -0.4566 to -0.06482) and *NGFR* fl/fl (left R^2 = 0.5046, 95% CI, -0.8389 to -0.08919 and right R^2 = 0.2793, 95% CI -0.8423 to 0.113) animals (**Supplementary Fig 3.4e and f**).

Finally, we performed RT-PCR to measure the relative knockdown of RNA transcript levels of *NTRK1* and *NGFR* following IT AAV9 Cre-eGFP. Unexpectedly, in *NTRK1* fl/fl animals treated with AAV9 Cre-eGFP there was a trend towards reduced expression but no significant difference in the expression level of *NTRK1* gene normalised to *GAPDH* 2 and 4 weeks post IT AAV9 Cre-eGFP. Compared to control at 1.01 ± 0.24 there was a FC of 0.7 ± 0.35 2 weeks post AAV9 Cre-eGFP and 0.47 ± 0.05 4 weeks post AAV9 Cre-eGFP in *NTRK1* expression. The level of *NGFR* expression in these animals remained unchanged. *NGFR* expression in controls was 1.00 ± 0.01 compared to 0.92 ± 0.12 2 weeks post AAV9 Cre-eGFP and 0.91 ± 0.12 4 weeks post AAV9 Cre-eGFP (**Fig 3.7b**). However, *NGFR* fl/fl animals showed a significant decrease in the expression of both *NTRK1* (p=0.0350) and *NGFR* (p=0.0092) genes, normalised to *GAPDH,* 2 weeks post AAV9 Cre-eGFP, one-way ANOVA with Tukey post-hoc. Expression of *NTRK1* decreased from a FC of 1.04 ± 0.4 in WT controls to 0.14 ± 0.17 2 weeks post AAV9 Cre-eGFP

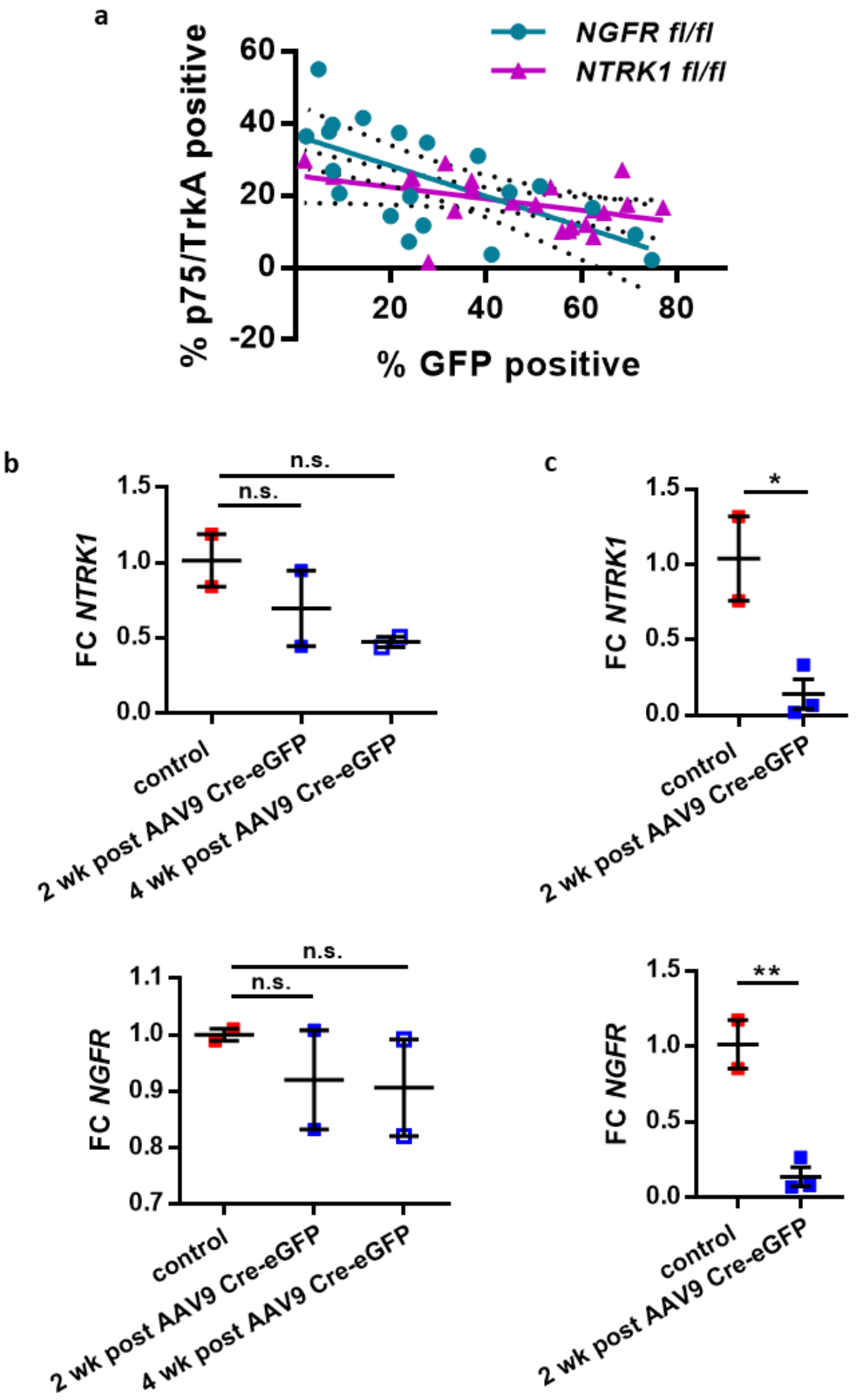

a
NGFR fl/fl
NTRK1 fl/fl
% p75/TrkA positive
% GFP positive
b
FC NTRK1
n.s.
n.s.
control
2 wk post AAV9 Cre-eGFP
4 wk post AAV9 Cre-eGFP
c
FC NTRK1
*
control
2 wk post AAV9 Cre-eGFP
FC NGFR
n.s.
n.s.
control
2 wk post AAV9 Cre-eGFP
4 wk post AAV9 Cre-eGFP
FC NGFR
**
control
2 wk post AAV9 Cre-eGFP

Figure 3.7 Intrathecal injection of AAV9 Cre-eGFP into NTRK1 fl/fl and NGFR fl/fl animals reduces expression of NTRK1 and NGFR at the RNA level in whole DRG from NGFR fl/fl animals but not in NTRK1 fl/fl mice. a- *Correlation of GFP expression and TrkA or p75 expression in NTRK1 fl/fl (R^2=0.1987) and NGFR fl/fl (R^2=0.4284) mice treated with AAV9 Cre-eGFP for 2 or 4 weeks respectively. Expression of NTRK1 and NGFR displayed as a FC normalised to C57Bl wildtype mice not treated with AAV9 Cre-eGFP in* ***b*** *NTRK1 fl/fl animals treated with AAV9 Cre-eGFP for 2 and 4 weeks, all tests not significant, and* ***c*** *NGFR fl/fl (NTRK1 p=0.0350, NGFR p= 0.0092) animals treated with AAV9 Cre-eGFP for 2 weeks. One-way ANOVA with Tukey post-hoc, n=2-6 animals.*

where expression of NGFR decreased from 1.01 ± 0.23 in WT controls to 0.14 ± 0.11 2 weeks post AAV9 Cre-eGFP (**Fig 3.7c**). This suggests that following knockout of *NGFR*, there is a downstream effect which results in a complementary decrease in *NTRK1*, which we discuss further below.

3.3.4 Knockout of TrkA using IT injection of AAV9 Cre-eGFP results in a decreased response to NGF mediated neurite outgrowth in MACS purified neurons

In order to test if the knockout of TrkA in neurons affects the neuronal response to NGF, *NTRK1* fl/fl animals were IT injected with AAV9 Cre-eGFP and all DRGs extracted 2 weeks post AAV9 Cre-eGFP injection. DRGs were triturated and both non-MACS and MACS purified neurons were grown for 24 hours in the presence or absence of NGF. Cell somas expressing GFP were considered to be successfully transduced and TrkA knockout was assumed for these cells. Non-MACS purified cultures did not show any significant difference in neurite outgrowth in either AAV9 Cre-eGFP negative or positive neurons grown in the absence of NGF, as observed by an average neurite outgrowth of 572 ± 339 µm in AAV9 Cre-eGFP negative

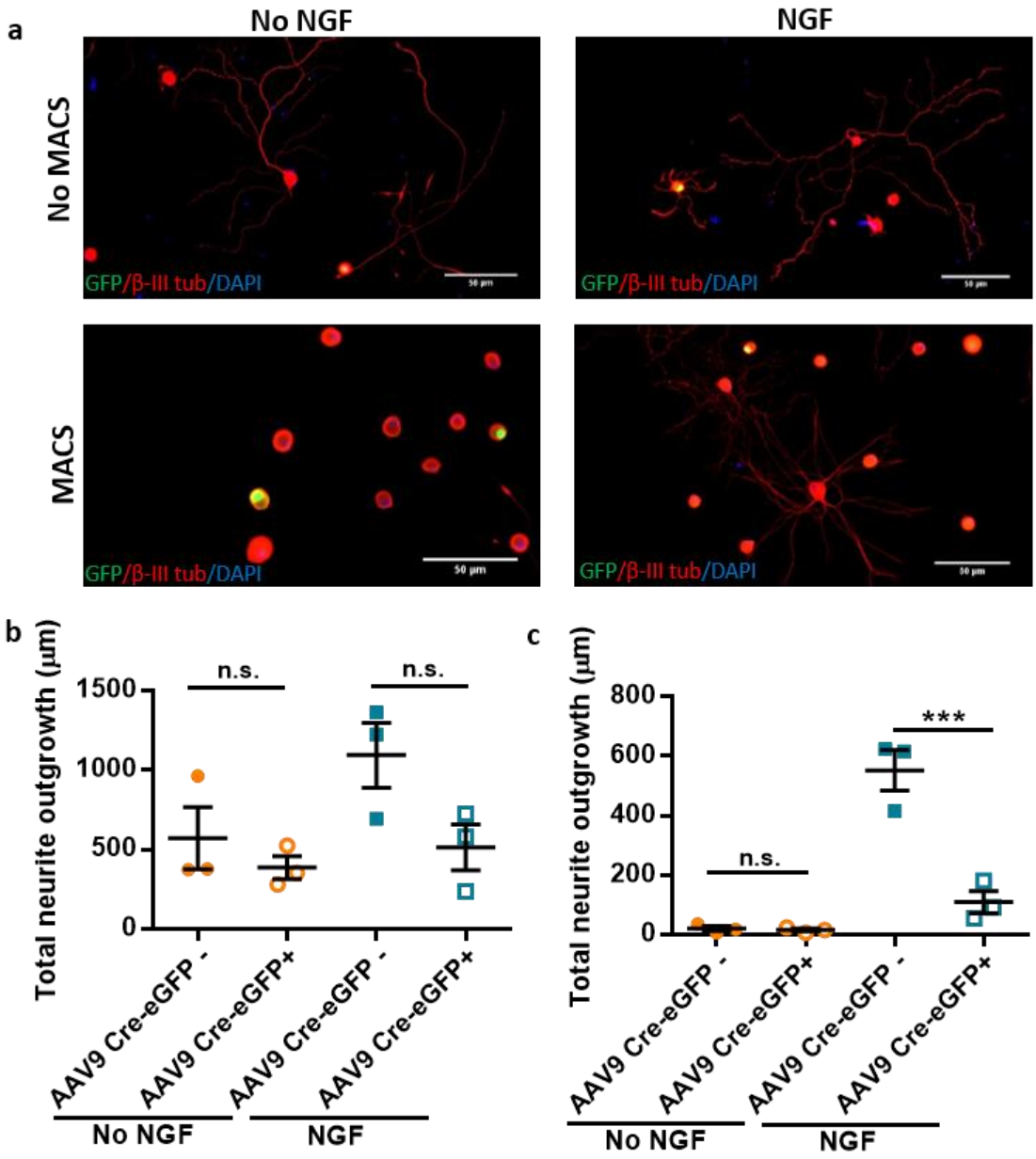


Figure 3.8 AAV9 Cre-eGFP mediated KO of NTRK1 reduces MACS purified neuronal response to NGF in vitro. a- *Representative ICC of non-MACS and MACS purified neurons from NTRK1 fl/fl mice two weeks post intrathecal injection with AAV9 Cre-eGFP. Quantification of neurite outgrowth following administration of 10ng/ml NGF of* ***b*** *non-MACS purified and* ***c*** *MACS purified neurons (p=0.0002). One-way ANOVA with Tukey post-hoc, n=3 individual cultures, 2 mice pooled per culture.*

compared to 387 ± 125 µm in AAV9 Cre-eGFP positive neurons. Furthermore, non-MACS purified cultures grown in the presence of NGF also showed no significant difference in neurite outgrowth, where the average neurite outgrowth in AAV9 Cre-eGFP negative neuron was 1094 ± 352 µm and Cre-eGFP positive neurons was 514 ± 251 µm (**Fig 3.8a and b**). This observation was also true for MACS purified cultures from IT injected animals grown in the absence of NGF, where there was no significant difference between an average neurite outgrowth of 20 ± 15 µm in AAV9 Cre-eGFP negative neurons compared to 16 ± 9 µm in AAV9 Cre-eGFP positive neurons (**Fig 3.8a and c**). However, in MACS purified cultures plated in the presence of NGF, there was a significant decrease in neurite outgrowth in MACS purified neurons following the knock-out of TrkA, p=0.0002, one-way ANOVA with Tukey post-hoc. AAV9 Cre-eGFP negative neurons showed an average neurite outgrowth of 552 ± 118 µm where AAV9 Cre-eGFP positive and therefore TrkA KO neurons showed an average neurite outgrowth of 109 ± 64 µm (**Fig 3.8a and c**). This same pattern for neurite outgrowth holds true for all other neurite outgrowth parameters, where there is a significant decrease in MACS purified neurons in GFP positive, TrkA KO, neurons treated with NGF compared to GFP negative controls (**Supplementary Fig 3.5 and 3.6**).

3.3.5 Knockout of p75 using IT injection of AAV9 Cre-eGFP results in a decreased response to NGF mediated neurite outgrowth in both non-MACS and MACS purified neuronal cultures

Similar to *NTRK1* fl/fl animals, *NGFR* fl/fl animals were given IT injection of AAV9 Cre-eGFP and 2 weeks later all DRGs were collected. DRG cultures of both non-MACS and MACS purified neurons were treated with and without NGF and the effect of p75 knock out on neurite outgrowth was measured after 24 hours *in vitro*. Cell soma with a GFP positive nucleus were defined as successfully transduced neurons with knock out of p75 assumed. In non-MACS

purified cultures there was no significant difference in the neurite outgrowth of both AAV9 Cre-eGFP negative and positive neurons grown in the absence of NGF, from an average overall neurite outgrowth of 2739 ± 380 µm in AAV9 Cre-eGFP negative neurons to 1077 ± 203 µm in AAV9 Cre-eGFP positive neurons. However, following NGF treatment we observed a significant decrease in the neurite outgrowth in non-MACS purified cultures. Non-MACS AAV9 Cre-eGFP positive p75 KO neurons showed an average neurite outgrowth of 2241 ± 260 µm compared to 5214 ± 1798 µm in AAV9 Cre-eGFP negative controls, p=0.0190, one-way ANOVA with Tukey post-hoc (**Fig 3.9a and b**). Similar to non-MACS there was no significant difference between AAV9 Cre-eGFP negative and positive neurons in MACS purified neurons cultured in the absence of NGF, from an average neurite outgrowth of 68 ± 54 µm in AAV9 Cre-eGFP negative neurons to 59 ± 48 µm in AAV9 Cre-eGFP positive neurons. However, comparable to non-MACS purified cultures there was a significant decrease in NGF mediated neurite outgrowth in MACS purified neurons with AAV9 Cre-eGFP positive neurons with p75 knockout showing an average neurite outgrowth of 301 ± 125 µm compared to 989 ± 327 µm in AAV9 Cre-eGFP negative controls, p=0.0066 one-way ANOVA with Tukey post-hoc (**Fig 3.9a and c**). This observation of a significant decrease in NGF mediated neurite outgrowth following knockout of p75 in both non-MACS and MACS cultures was also found in other neurite outgrowth parameters (**Supplementary Fig 3.7 and 3.8**). The only exception to this being in MACS purified neurons treated with NGF where there was no significant difference in the length of the shortest neurite between the AAV9 Cre-eGFP negative controls and AAV9 Cre-eGFP positive neurons, one-way ANOVA with Tukey post-hoc (**Supplementary Fig 3.8**). Interestingly, in non-MACS purified neurons plated in the absence of NGF there was a significant decrease in the longest neurite (p=0.0026), mean neurite length (p=0.0478), number of neurites per neuron (p=0.0075) and the total length from the longest branch (p=0.0410), one-way ANOVA with Tukey post-hoc (**Supplementary Fig 3.7**). This suggests that although there was no significant

difference in the total neurite outgrowth of neurons, these other neurite outgrowth parameters suggest that there may be a difference in the neurite outgrowth of p75 knockout neurons, even in the absence of NGF.

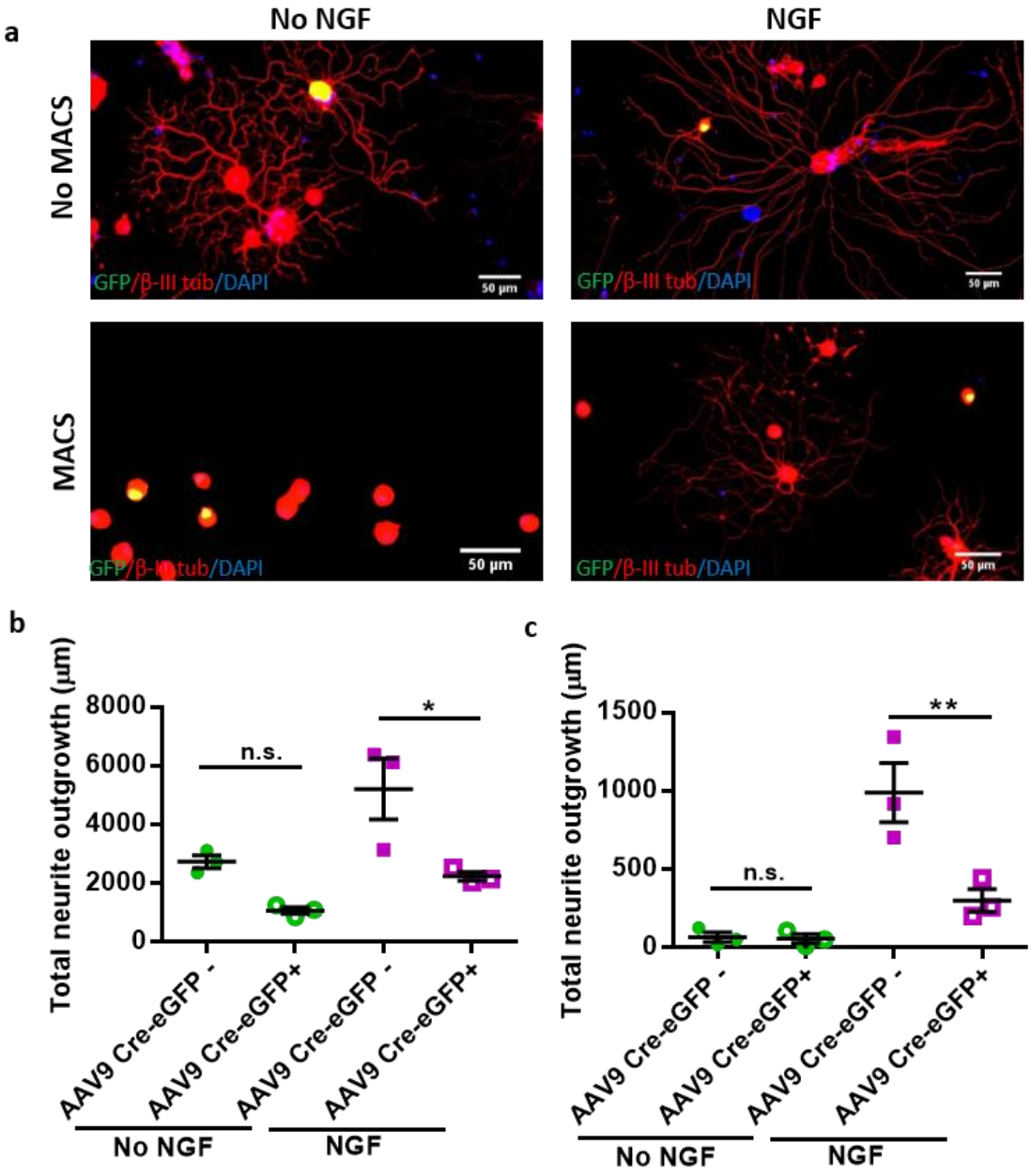


Figure 3.9 AAV9 Cre-eGFP mediated KO of NGFR reduces MACS purified neuronal response to NGF in vitro. a- *Representative ICC of non-MACS and MACS purified neurons from NGFR fl/fl mice two weeks post intrathecal injection with AAV9 Cre-eGFP. Quantification of neurite*

*outgrowth following administration of 10ng/ml NGF of **b** non-MACS purified (p=0.0190) and **c** MACS purified neurons (p=0.0066). One-way ANOVA with Tukey post-hoc, n=3 individual cultures, 2 mice pooled per culture.*

3.4 Discussion

3.4.1 Lenti Cre-GFP viral mediated knockout of TrkA or p75 *in vitro* is not successful

To investigate the effect of knockout of either TrkA or p75 *in vitro* we decided to isolate DRG neurons from *NTRK1* fl/fl and *NGFR* fl/fl mice respectively and optimise a culture protocol to introduce Cre recombinase to neurons using a lenti viral vector. Firstly, we tested the transduction efficiency of several lenti viruses encoding Cre-GFP fusion protein in non-MACS purified neurons isolated from WT C57Bl mice. Cultures were grown in BS medium and treated with lenti virus at varying MOI directly in the medium for up to 5 days. Cells were daily monitored for the expression of GFP in the neuronal cell somas. Transduction and expression of Cre-GFP is usually observed from 24-48 hours in culture (Millington et al. 2009; Denning et al. 2013).

The first lenti-viral vector tested was purchased from Amsbio (LVP576) which encoded Cre recombinase with a GFP-puromycin selection marker under the CAG promoter. After 2 days in culture no expression of GFP was observed, therefore to test if this was due to a defective virus or due to a weak signal from GFP this virus was tested in non-MACS purified cultures isolated from TdTomato mice. These particular TdTomato mice contained a TdTomato-flox-STOP-flox cassette where there is a translational stop upstream of the TdTomato gene flanked by loxP sites. In the presence of an active Cre recombinase the STOP cassette is removed and

there is expression of the TdTomato protein. Treatment of non-MACS purified cultures from TdTomato mice with Amsbio lenti Cre-GFP however failed to induce expression of the TdTomato signal in addition to a failure to observe signal from the GFP marker suggesting that the purchased vector was not functional for our purpose. We repeated this experiment with a new virus from a different batch however the result was the same.

Testing of a different lenti Cre-GFP from Cellomics Technology which encodes a Cre-GFP fusion protein under the control of the CMV promoter in non-MACS purified DRG cultures from C57Bl6 WT mice however showed expression of GFP in neuronal cell somas after 2 days in culture. To increase the number of neurons expressing GFP we optimised our protocol. Instead of adding the lenti virus directly to the plating medium we pre-incubated the virus with the cell suspension before plating. Cultures from TdTomato mice after 2 days in culture incubated with the virus showed expression of both GFP and TdTomato marker proteins from all MOI of virus showing that there was effective transduction of virus in a higher proportion of cells compared to the previous method of viral transduction and proved the Cre recombinase protein was active.

In order to optimise the transduction of MACS purified DRG cultures with lenti Cre-GFP, we tested a number of different MOI ranging from 20-50 for 2-5 days in MACS purified cultures. We observed that there was the highest percentage of transduced cells using a MOI 20 or 30 for 3 days or a MOI 50 for 3, 5 and 7 days. However, in cultures grown with the virus for 5 and 7 days or treated with MOI 50 lenti virus there was a larger amount of debris present in the culture suggesting that in these cultures there was cell death likely due to toxic exposure to the lenti virus. We concluded from this that either MOI 20 for 3 days or MOI 30 for 3 days is the optimal incubation time for the transduction of MACS purified neurons. For all subsequent experiments we decided to use a MOI 30 for 3 days in MACS purified cultures to allow maximal transduction and time for the Cre recombinase to be activated and knockout expression of the

floxed gene. However, it shall be noted that when we evaluated the cultures after fixation, we found a greater number of cells on average remaining in cultures treated with lenti Cre-GFP at MOI 20 for 3 days.

For our RT-PCR experiments we designed and tested RT-PCR primers upstream, over and downstream of the floxed regions of the NTRK1 gene in order to investigate if there was complete KO of the floxed gene, or if there was partial transcription of mRNA for both the *NTRK1* fl/fl and *NGFR* fl/fl cells following exposure to Cre recombinase. However, only one of the tested primer pairs were efficient (**Supplementary table 3.1 and 3.2**). RT-PCR analysis of the *NTRK1* and *NGFR* expression following lenti Cre-GFP mediated knockout in *NTRK1* fl/fl or the *NGFR* fl/fl cell cultures showed no difference in the expression of either gene after treatment with MOI 30 for 1-3 days. We ensured that the quality of the RNA was high by testing it with an Agilent bioanalyser. As expected there was some cell loss in samples collected after 3 days in culture compared to control samples untreated with lenti Cre-GFP collected straight off the MACS LD column. Despite the reduction in the number of cells with the increased number of days in culture there was still sufficient amount of RNA for amplification, using the limit of the amplification capacity of this commercial kit (Quantitect whole transcriptome kit, Qiagen). However, this amplification method has since been shown in the lab to not sensitively amplify small amounts of RNA at this limit compared to other more specialised amplification kits (Repli-g WTA single cell amplification kit, Qiagen kit, data not shown, performed by post-doctoral researcher Dr Damini Tewari). In conclusion, we are not sure whether the lack of change in the expression of the *NTRK1* or the *NGFR* genes following lenti Cre-GFP mediated knock out is the result of improper amplification of RNA. This could be tested by repeating this experiment the now identified better kit for RNA amplification and cDNA conversion. However, this was beyond the scope of this study.

Another explanation for the failure of gene knockout might be that 3 days in culture with the lenti virus was not sufficient. Typically it can take 1-2 days for transduction and expression of viral genes which could result in a lag time between expression of the Cre recombinase and the knock out of the gene. However, we based our time points on the expression of TdTomato in the TdTomato-flox-STOP-flox strain that showed knockout of the stop cassette. Also, our experiments showed that cultures incubated with the virus for more than three days were less viable. Although our pilot experiments showed that knockout should have occurred after 3 days in culture the RNA stability is unknown. Therefore, we might be measuring stable RNA rather than newly expressed RNA. Furthermore, even if there was gene knockout after incubation with the virus for three days, at this point we don't know if this had resulted already in protein knockout. The turnover rate of the two proteins are not known to us. Any residual protein in the cell and stored in vesicles (Lessmann, Gottmann, and Malcangio 2003) could still be functional and account for the similar neurite outgrowth in control and knockout cells.

Despite there being no knockout in *NTRK1* or *NGFR* as measured by RT-PCR, due to a number of reasons mentioned above there was reason to not fully trust this result. Therefore, we investigated further the neuronal response to NGF following of knock out of either the TrkA receptor using MACS purified cultures from *NTRK1* fl/fl mice or the p75 receptor from MACS purified cultures from *NGFR* fl/fl mice. We treated MACS purified cultures with NGF either at the time of isolation and start of culturing in combination with lenti Cre-GFP or after 1 or 2 days in culture with the lenti Cre-GFP. There was no significant difference, except for those mentioned below, in neurite outgrowth following application of NGF at any time point in both cultures from both *NTRK1* fl/fl and *NGFR* fl/fl mice. In *NTRK1* fl/fl mice there was a significant increase in neurite outgrowth following in cultures treated with NGF 1 and 2 days after plating compared to cultures treated at the time of isolation. This suggests that after TrkA knockout

neurons were able to respond better to NGF than when plated in NGF throughout the 3 days left in culture. Additionally, this observation could be the result of interference between NGF and the virus which could result in inhibition of NGF signalling. Similarly, addition of virus to MACS purified cells could induce cellular stress and therefore neurons would not respond to NGF as predicted. Another explanation might be that after TrkA knockout there is a compensatory mechanism upregulating p75 expression, which then mediates the NGF effect, however we were not able to confirm this on RNA level using RT-PCR.

In MACS purified cultures from *NGFR* fl/fl mice treated with NGF and lenti Cre-GFP to knockout the p75 receptor there were no significant differences in neurite outgrowth between either the knockout or controls, or between cultures treated with NGF at different times points (0, 1 or 2 days). We observed in control (no virus treatment) cultures from both *NTRK1* fl/fl and *NGFR* fl/fl mouse strains that the MACS purified neurons showed no significant difference in neurite outgrowth between cultures treated with NGF for the entire 3 day duration of the experiment or untreated controls. In the previous chapter (chapter 2) we described that differences in neurite outgrowth assays were observed after 1 day in culture (**Fig 2.6**). Investigating neurite outgrowth after *NTRK1* or *NGFR* knockout and NGF treatment did not reveal any NGF induced differences at the time of plating, 1 or 2 days after treatment. This might suggest that the neurons themselves secrete enough neurotrophins to sustain and saturate neurite outgrowth.

In summary, our conclusion from this series of somewhat frustrating experiments was: delivery of the Cre recombinase to the floxed neurons via lenti virus is not the right approach since the knockout takes too long and the cells are not healthy anymore by the time of successful knockout.

3.4.2 IT injection of AAV9 Cre-eGFP was effective in knocking out expression of floxed genes but there was variation in the transduction patterns

After finding that transduction of MACS purified cultures from *NTRK1* fl/fl and *NGFR* fl/fl mice was ineffective we decided to use an *in vivo* approach. We used intrathecal (IT) injections with an AAV9 Cre-eGFP into *NTRK1* fl/fl and *NGFR* fl/fl mice to knockout expression of TrkA and p75 respectively. IT injections with AAV9 is a well-established technique in the lab. We used an IT cannula to inject AAV9 into the intrathecal space using a method developed by a PhD student in the lab, Nikita Khovanov. All injections in this study were performed by Nikita Khovanov.

Firstly, we measured the effectiveness of the knockout of the TrkA and the p75 mice in the respective floxed mouse lines. We observed that in the both *NTRK1* fl/fl and *NGFR* fl/fl mice injected with AAV9 Cre-eGFP there was expression of GFP 2 and 4 weeks after injection with AAV9 Cre-eGFP. Interestingly, we observed that after 4 weeks there were less transfected cells and lower GFP expression compared to 2 weeks. We found this trend in both mouse lines, but in the *NTRK1* fl/fl mice it reached significance. This observation is counter-intuitive. However, it has been found that overexpression of GFP in some cases is toxic for cells, especially when highly expressed (H. S. Liu et al. 1999). This phenomenon could explain the reduced efficiency after 4 weeks, however we did not detect any evidence of cell death in our 4 week samples (L4 DRG sections). The loss in the signal of GFP could be the result of the Cre-eGFP fusion protein being degraded after active cleavage of the loxP sites in the gene of interest by the Cre recombinase-GFP fusion protein (Schulz et al. 2007), however as the virus would continue to produce the Cre-eGFP fusion protein in transfected cells this is unlikely. Next, we verified knockout of TrkA by staining for the protein in *NTRK1* fl/fl L4 DRG neurons injected with AAV9 Cre-eGFP. We found in both time points a comparable reduction in the percentage of TrkA expressing cells. However, the reduction was not complete due to insufficient transfection. As

assessed by expression there is only about 60% transduction efficiency at the peak of transduction 2 weeks after injection. Similar to *NTRK1* fl/fl mice, expression of the GFP marker protein in L4 DRGs from *NGFR* fl/fl mice treated with AAV9 Cre-eGFP correlated with a decrease in the percentage of neurons expressing the p75 receptor compared to control levels, however this was not significant. Contrary to the *NTRK1* fl/fl mice there was a further decrease in the expression of p75 after 4 weeks incubation with AAV9 Cre-eGFP compared to 2 weeks after injection despite there being a decreased number of neurons expressing the GFP marker protein 4 weeks after injection. The reason for this discrepancy in knockout between *NTRK1* fl/fl and *NGFR* fl/fl animals 2 and 4 weeks after IT injection of AAV9 Cre-eGFP is likely the result of large variation in the cannula placement during the injection, which in some animals results in unilateral transduction as observed in this study. As only 30% of *NGFR* fl/fl animals (compared to 91% of *NTRK1* fl/fl animals) show a side bias in transduction and the percentage of cells expressing p75 are calculated across both L4 DRG, a higher proportion of cells overall could be transduced in *NGFR* fl/fl animals which resulted in this further decrease in p75 expression.

In both *NTRK1* fl/fl and *NGFR* fl/fl mouse strains we observed a negative correlation between the percentage of cells expressing GFP and the percentage of cells expressing either TrkA or p75 from the respective knockout mouse. This indicates that as the percentage of neurons expressing of GFP increased, indicating successfully transduction, the percentage of neurons expressing the protein of interest decreased suggesting successful knockout of the targeted gene. TrkA and p75 NGF receptors are known to be only expressed in certain populations of neurons (Chapter 1.1) (Aloe et al. 2012; Richner et al. 2014). We observed the same when we stained sections of L4 DRGs from WT mice without IT injection and found that only 40% of neurons expressed of TrkA or p75. We established that there is only 60% transduction of DRG. When we consider that amongst the 60% many will be negative or positive for TrkA or p75, we

can also assume that a proportion of the cells not transduced will also be positive or negative for TrkA or p75. This therefore may account for the incomplete knockout of either TrkA or p75 following IT injection of AAV9 Cre-eGFP. However, a decrease in the expression of either the TrkA or the p75 receptor with an increase in the expression of GFP shows that there is no preferential transduction of certain neurons, there is at least partial transduction of the neurons of interest. Furthermore, work by a PhD in the lab Nikita Khovanov has shown that there is no preferential transduction of specific neurons in regards to cell size as he has observed that there was an equal level of transduction across small, medium and large diameter neurons (data not shown).

We then used RT-PCR to confirm knockout of genes in whole DRGs taken from floxed animals IT injected with AAV9 Cre-eGFP. We measured the expression of both *NTRK1* and *NGFR* gene expression in injected *NTRK1* fl/fl animals. Interestingly, we found that despite a trend towards a decrease in expression there was no significant decrease in the expression of *NTRK1* either 2 or 4 weeks after injection. This is likely due to RNA being extracted from whole L3 DRG pooled from both sides and therefore these samples contain a mix of neuronal and non-neuronal cells. In addition we only expect a proportion of neurons to be transduced with AAV9 Cre-eGFP and therefore be knockout for TrkA. We also measured the expression of *NGFR* 2 and 4 weeks after injection and observed no significant difference compared to whole DRGs from WT C57Bl animals which were not injected, which is again likely the result of the confounds of a mixed sample from whole DRGs as mentioned above. It should also be noted that these changes in gene expression from *NTRK1* fl/fl animals were only calculated using 2 independent samples since a third sample failed in the isolation process as there was insufficient material to convert to cDNA. Therefore, without a third sample no statistics can be performed from this experiment. Following investigation of *NTRK1* fl/fl animals we then measured the change in expression of *NTRK1* and *NGFR* in AAV9 injected *NGFR* fl/fl. We found that 2 weeks after IT

injection there was a significant decrease in the expression of *NGFR* confirming that AAV9 Cre-eGFP mediated knockout of the p75 gene was effective. Interestingly, we also observed a significant decrease in the expression of the TrkA gene *NTRK1* in these animals. The p75 receptor has been identified to control apoptosis, but in combination with TrkA it has also been shown to contribute to growth and survival of neurons. This loss in both receptors could be the result of neuronal death following knock out of the *NGFR* gene, however as previously mentioned there is no evidence for a loss in the number of neurons in L4 DRG used for immunohistochemistry however, this could be further confirmed indirectly by measuring the cell size distribution of the remaining neurons to see if there is loss of a particular population of neurons. The decrease in the expression of the *NTRK1* gene could be directly related to the loss of the *NGFR* gene and therefore could be regarded as a downstream effect following knockout of p75. It should also be noted that there is no data for gene expression of *NTRK1* or *NGFR* genes 4 weeks after IT injection. This was due to a failure to isolate sufficient RNA from whole DRG in these samples and therefore would need to be repeated with a second cohort of animals in the future. For the future, more experimental replicates will be needed. However, we also propose to change the method used to isolate the cells in order to improve the assessment of changes in RNA expression. Following collection and dissociation of DRGs from IT injected neurons, transduced neurons should be isolated using FACS and RNA isolated from these neurons. Non-transduced cells without a GFP tag could be collected and used as an internal control, however, this would also contain non-neuronal cells which would dilute the expression of the gene of interest. One solution would be to use MACS purification to isolate neurons prior to FACS isolation, however, this would limit the GFP neurons to contain only small diameter neurons as medium and large diameter neurons are lost during MACS purification.

3.4.3 MACS purified neurons following knockout of either the TrkA or the p75 receptor using IT injection of AAV9 Cre-eGFP are irresponsive to NGF treatment in culture

In order to determine if knockout of either the TrkA or p75 NGF receptors has an effect on the neuronal response to NGF we cultured non-MACS and MACS purified neurons from *NTRK1* fl/fl and *NGFR* fl/fl mice2 weeks after IT injection of AAV9 Cre-eGFP. We made the assumption that all GFP expressing neurons were positively transduced and knockout for the gene of interest. Non-GFP expressing cells were used as internal controls for WT neurons. Cultures isolated from AAV9 injected *NTRK1* fl/fl animals and MACS purified showed a significant decrease in the neurite outgrowth following NGF treatment in neurons with knockout of TrkA compared to controls suggesting that TrkA is essential for NGF mediated neurite outgrowth in pure neurons. However, non-MACS purified treated with NGF showed a trend towards a decrease in neurite outgrowth, however, the difference was not significant. This could be due to non-MACS cultures secreting factors such as neurotrophins which can induce neurite outgrowth through signalling via other growth factor receptors e.g. BDNF. In cultures isolated from injected *NGFR* fl/fl animals and MACS purified we found that neurons treated with NGF show a significant decrease in neurite outgrowth following knockout of p75 compared to controls similar to MACS purified cultures with knockout of TrkA treated with NGF. Interestingly, non-MACS cultures treated with NGF also showed a significant decrease in neurite outgrowth in neurons with knockout of p75 compared to controls. This suggests that p75 is essential for NGF mediated growth in culture. Taken together these results where both knockout of TrkA or p75 in neurons results in a decrease in neurite outgrowth is contradictory to the common belief that alone TrkA signals growth or survival of neurons where p75 signals apoptosis of cells as both receptors appear to play a role in neuronal growth response to NGF (Capsoni and Cattaneo 2006; Meeker and Williams 2014). P75 is said to be a promiscuous neurotrophin

receptor for NGF, BDNF and NT-3. It could be that the significant decrease in neurite outgrowth in non-MACS purified cultures from *NGFR* fl/fl animals compared to *NTRK1* fl/fl is the result of a loss in not only NGF signalling but also BDNF and NT-3 signalling. BDNF and NT-3 are secreted from neuronal and non-neuronal cells (Richner et al. 2014; Lessmann, Gottmann, and Malcangio 2003). In the MACS purified cultures however it is likely that the decrease in neurite outgrowth is sole effect of the knockout of p75 on NGF signalling as in these cultures there are predominately small diameter nociceptors, known to be approximately 50% peptidergic nociceptors which express the TrkA receptor. TrkB (receptor for BDNF) is mainly expressed by medium diameter neurons and TrkC (receptor for NT-3) is expressed mainly by large diameter neurons (Richner et al. 2014). As previously described (chapter 2.4), medium and large diameter neurons are absent from MACS purified cultures and MACS purified cultures exhibit a lag in neurite outgrowth due to the lack of supportive factors secreted from non-neuronal cells. Therefore, we can assume that the increase in neurite outgrowth in MACS purified control cells treated with NGF compared to neurons cultured in the absence of NGF is the result of NGF mediated neurite outgrowth. As a result, the decrease in MACS purified cells with knockout of p75 cultured in the presence of NGF is the result of an insensitivity of neurons to NGF mediated by a loss in p75.

3.4.4 Future directions for AAV9 Cre-eGFP mediated knockout of TrkA or p75

We tried to knockout *NTRK1* or *NGFR* by isolating and MACS purifying neurons from *NTRK1* fl/fl and *NGFR* fl/fl mouse strains and treating them with lenti Cre-GFP virus to introduce Cre recombinase and achieve knockout of TrkA and p75 gene respectively *in vitro*. However, we concluded from the results that this method is not suitable for our study. Therefore, we chose to use AAV9 Cre-eGFP injected IT to effectively transduce DRG neurons *in vivo*. However, we observed that there was a varying degree of unilateral transduction as assessed by expression of the GFP marker protein. In *NTRK1* fl/fl injected animals there was evidence of a unilateral

bias in the L4 DRG in 91% of animals (10 out of the 11 animals injected), however, only approximately 30% of *NGFR* fl/fl showed unilateral preference for transduction. The unilateral nature of the injection is likely the result of the cannula placement during the injection. In order to avoid damage to the spinal cord, the cannula is inserted on the left-hand side of the spinal cord, rather than the midline. Due to the observed bias in the *NTRK1* fl/fl DRGs, the injection of the *NGFR* fl/fl animals was slightly altered as the end was pointed towards the midline during injection. The improvement of the injection resulted in only 30% animals showing any bias. This is considerably better than 91% achieved before, however, it is not yet ideal since the aim is to have 0% bias. Any bias would complicate any behavioural experiments for obvious reasons and normalisation to the degree of knockout of left or right would have to be performed. However, if a behavioural effect was observed, or not, various degrees of transduction could elucidate if there was any kind of dose response.

In order to perform behavioural experiments, it would be beneficial to have better controls for the study. The control used in our study used WT C57Bl mice (brought in from Harlan, UK) which were not injected with any AAV9. We chose this control as the transgenic mice are all bred onto the same C57Bl background. Ideally, this study would have used littermate controls that were WT for the floxed alleles. However, the number of available animals was limited due to breeding constraints. Therefore, we opted to use commercially supplied control animals in this pilot experiment to determine if using an AAV9 viral vector resulted in any significant knockout of either the *NTRK1* or the *NGFR* gene from *NTRK1* fl/fl and *NGFR* fl/fl animals respectively. In order to further validate this model, particularly for use in behavioural experiments, a number of additional controls groups should be needed included, such as naïve mice or sham groups, saline injected, injected with an AAV9 GFP control virus and finally the AAV9 Cre-eGFP injected group. These controls would shed light on any effect caused by the injection from the cannula itself (potential spinal damage), viral transduction or presence of

the floxed allele itself. Such in-depth analysis of the model was outside of the scope of this thesis due to animal and time constraints.

In this study we used non-MACS and MACS purified cultures from AAV9 Cre-eGFP injected and measured neurite outgrowth response to 24 hours of NGF treatment. It is important to note that there are confounds to the neurite outgrowth method used in this study that could be improved in the future. Following IT injection of AAV9 Cre-eGFP there is incomplete transduction of DRG neurons which were then MACS purified and used for cultures. As these cultures contain a mix of peptidergic and non-peptidergic neurons it is therefore it is reasonable to assume that for example in the case of cultures from *NTRK1* fl/fl animals a proportion of the GFP negative neurons analysed for neurite outgrowth are peptidergic and TrkA positive internal controls. However, there would also be a subset of these neurons which are non-peptidergic and therefore neurons which would not express TrkA and are unsuitable as controls. Similarly, a proportion of the GFP positive neurons would be peptidergic neurons which are the targeted KO neurons for TrkA where other neurons would be non-peptidergic neurons which would not express TrkA and confound the results of this study. Therefore, in the future it would be advisable to counterstain these cultures for both a non-peptidergic marker such as Ret or IB4 in addition to a counterstain for either TrkA or p75 positive neurons for cultures from *NTRK1* fl/fl and *NGFR* fl/fl mice respectively. In this case neurons that are stained positive for either TrkA or p75 and negative for GFP would be used as control neurons where neurons which are positive for GFP and negative for the non-peptidergic marker would be analysed as KO neurons in the neurite outgrowth assay.

Other assays that could be used to measure a potential effect of the knockout of the TrkA or p75 receptor. These include calcium imaging and patch clamping of GFP negative and positive neurons. In calcium imaging NGF has been used as a sensitising agent for neurons. Bonnington *et al* described how the use of repetitive treatments of embryonic DRG cultures with capsaicin

gradually desensitised the neuronal response to the stimulus as seen by a decreased calcium influx. However, when they performed five applications of capsaicin and then added NGF to the culture, a sixth application of capsaicin then produced a response similar to the first application of the stimulant suggesting that the NGF had acted to sensitise the neurons to the stimulus (Bonnington and McNaughton 2003). We used the calcium imaging method described in Bonnington *et al* in MACS purified DRG cultures from adult mice however we were not able to reproduce the described results. In our hands only approximately 10% of neurons became re-sensitised by repeated capsaicin and NGF treatment (data not shown). Potential explanations why we were not able to reproduce the published data might be that we used adult DRG cultures as opposed to embryonic cultures or that we used MACS purified cultures as opposed to whole DRG cultures. Another important difference to note is that the Bonnington *et al* study cultured DRG neurons in the presence of NGF as embryonic neuronal cultures require NGF for survival and therefore this pre-exposure to NGF may have primed cells for this response. Since we are interested the response of cells to NGF following the KO of either the *NTRK1* or *NGFR* gene from floxed animals, we chose not to use any pre-incubation with NGF in these cultures. However, for the future pre-sensitisation of our cells to NGF could be performed and the assay repeated. Another approach, as mentioned above, would be patch clamping, a method used to investigate and characterise the biophysical properties of neurons. We could employ it to further characterise our MACS purified knockout cells. Tsantoulas *et al* used patch clamping in DRG cultures 6-8 days after culturing cells in microfluidic chambers where the cell soma and axons are cultured in separate compartments. In this study cell somas were patched and current was constantly measured in the cell soma following pulses of electrical stimulation to the axonal compartment. Using micropipettes, patch clamping also allows application of pharmacological agents such as lidocaine or neurotrophins, such as NGF, directly to the cell being recorded (Tsantoulas et al. 2013).

Performing patch clamping on our knockout cells could identify if NGF stimulation of TrkA or p75 knockout cells results in a diminished firing pattern and would prove that NGF stimulation is essential for the electrophysical potential of pure DRG neurons following application of ligands such as capsaicin.

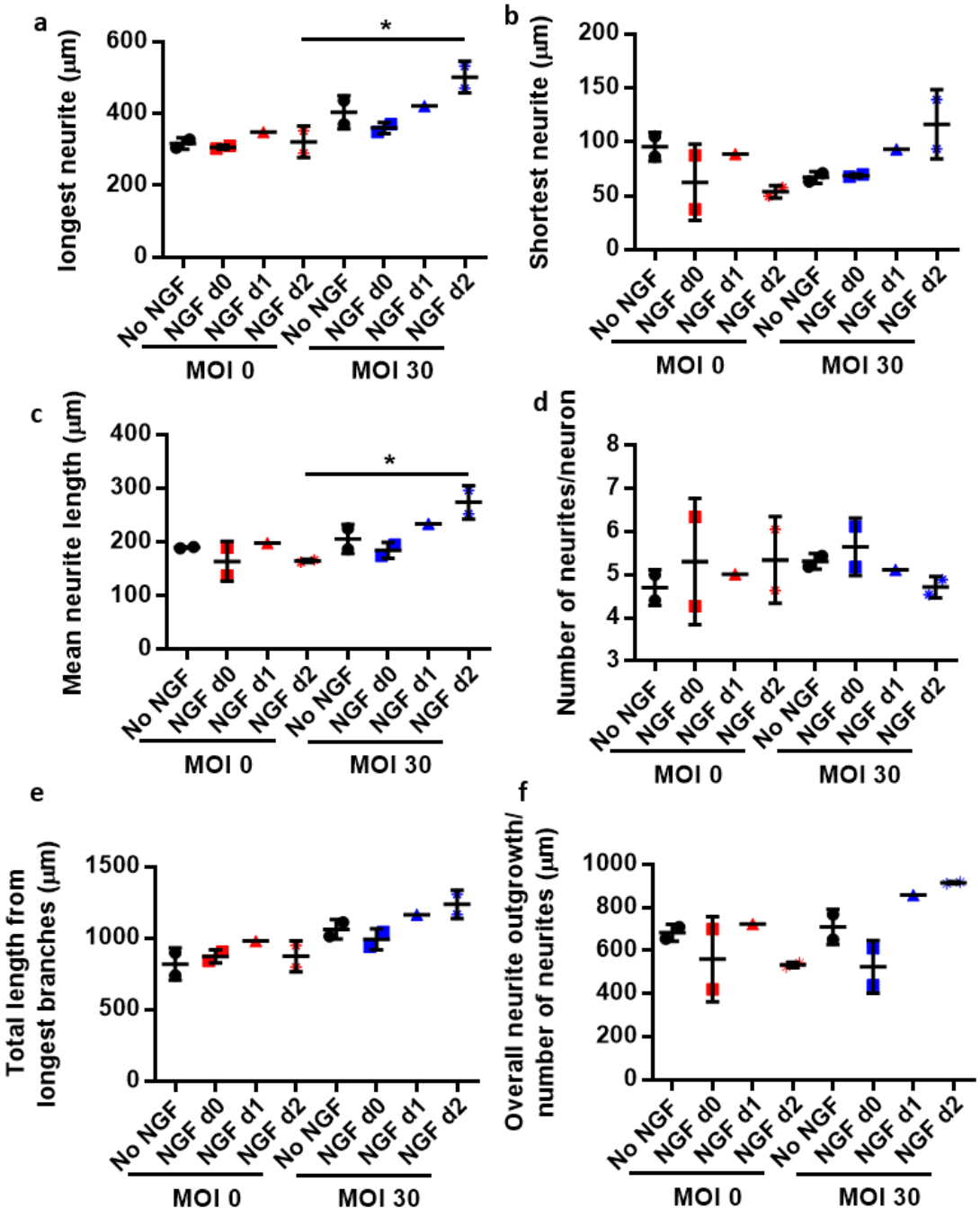
3.5 Supplementary material

Primer pair	Ntrk1 qPCR primers	Primer sequence	Efficency of primer pair	Single peak melt curve?	Positive band on agarose gel	Useful primer pair?
1	qPCR_Ntrk1 start_F_n1	TGTCGCGAGGTCTGCT	?	x	x	x
	qPCR_Ntrk1 start_R_n1	TTGCAGGTGCTGCTGGTT				
2	qPCR_Ntrk1 start_F_n2	CTCCGCCGCATCCTGT	?	x	x	x
	qPCR_Ntrk1 start_R_n2	AGGTGCTGCTGGTTTTCC				
3	qPCR_Ntrk1 over_F_n1	CAGATGCCTTCCGTTTCACC	2.425	Y	Y	Y/N
	qPCR_Ntrk1 over_R_n1	GAGGCCCTGCACAGTTTTCC				
4	qPCR_Ntrk1 over_F_n2	GAGTGGCCTCCGCTTTGT	2.215	Y	Y	Y
	qPCR_Ntrk1 over_R_n2	GCACAGTTTTCCAGGAGAGGG				
5	NTRK1_qPCR_KO_F_over flox	AGATGCCTTCCGTTTCACCC	2.1	Y	Y	Y
	NTRK1_qPCR_KO_R_over flox	GAGAGGCCCTGCACAGTTTT				
6	NTRK1_qPCR_KO_F_after flox	ACGCATGTCAACAACGGGAA	1.88	Y	Y	Y
	NTRK1_qPCR_KO_R_after flox	CATAAAAGCAGCCATGACGGAG				
7	qPCR_Nrtk1 after_F_n1	GAAGAATGTGACGTGCTGGG	1.932	Y	Y	Y
	qPCR_Nrtk1 after_R_n1	GAAGGAGACGCTGACTTGGA				
8	qPCR_Ntrk1 end_F	ATATCTAGCCAGCCTGCACTTTGT	2.599	Y	Y	Y/N
	qPCR_Ntrk1 end_R	TGCTCATGCCAAAGTCTCCA				

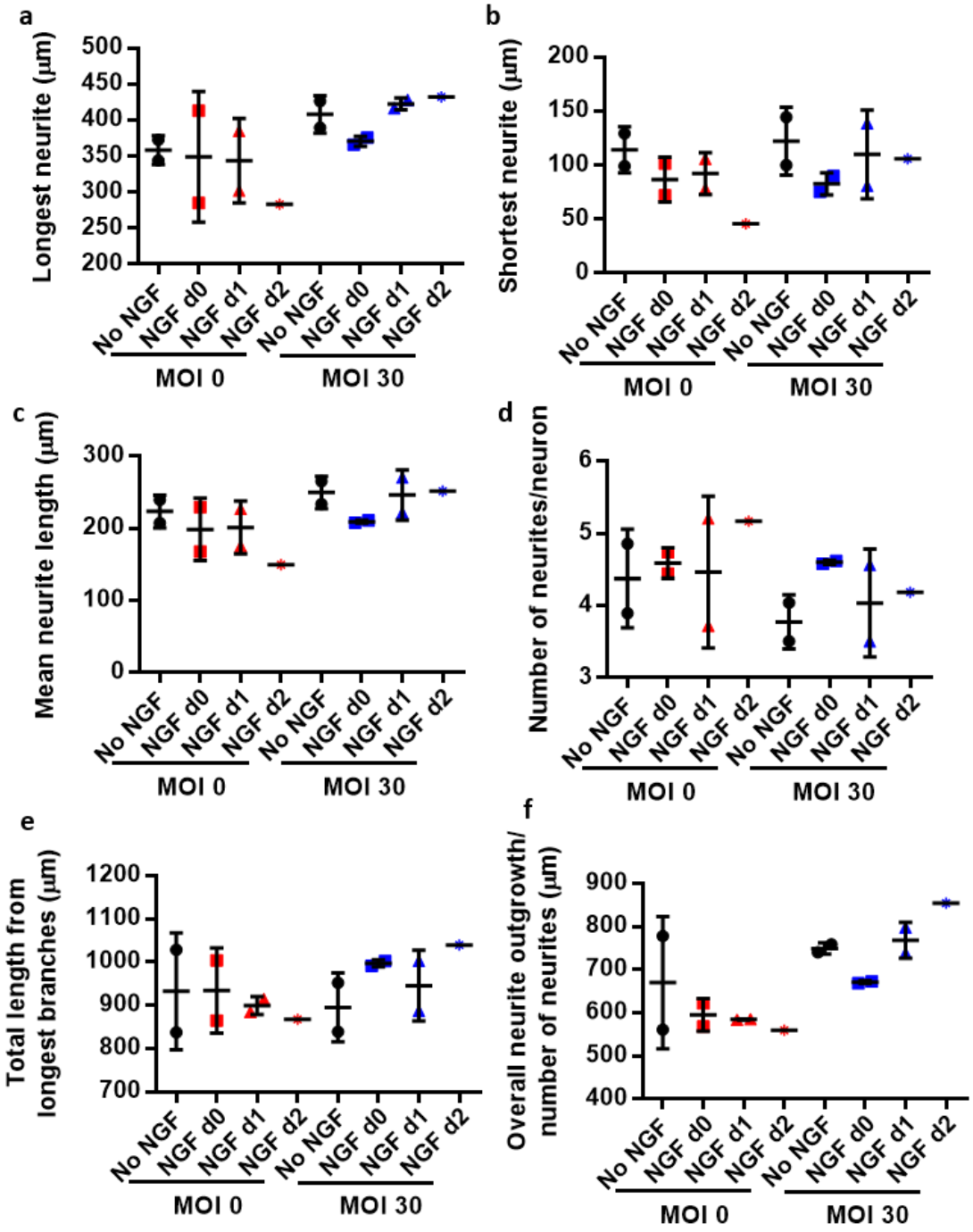
Supplementary table 3.1 NTRK1 RT-PCR primers tested, highlighted is the primer pair used in subsequent experiments

Primer pair	NGFR	Primer sequence	Efficency of primer pair	Single peak melt curve?	Positive band on agarose gel	Useful primer pair?
1	qPCR_Ngfr start/across_F_n1	TTGCGGAGCCAACCAGACC	2.11	Y	Y	Y
	qPCR_Ngfr start/across_R_n1	CCACACAGGGAGCGGACATAC				
2	NGFR_qPCR_KO_F_after flox	TGGGCTGACGCCGAATG	x	x	x	N
	NGFR_qPCR_KO_R_after flox	TGGCTATGAGGTCTCGCTCT				
3	NGFR_qPCR_KO_F_end seq	GGCCTTGTGGCCTATATTGCT	x	x	x	N
	NGFR_qPCR_KO_R_end seq	CTGTCGCTGTGCAGTTTCTCT				
4	qPCR_Ngfr end_F_n1	CCGCTGACAACCTCATTCC	1.999	Y	Y	Y
	qPCR_Ngfr end_R_n1	GGCTGTTGGCTCCTTGTTTATTT				
5	qPCR_Ngfr end_F_n2	TGTCTATTGCTCCATCTTGGCT	1.865	Y	Y	Y
	qPCR_Ngfr end_R_n2	CCGGCTGTTGGCTCCTTG				
6	qPCR_Ngfr end_F_n3	GGCCTTGTGGCCTATATTGCTT	2.204	N	x	N
	qPCR_Ngfr end_R_n3	CGCTGTCGCTGTGCAGTTTC				

Supplementary table 3.2 NGFR RT-PCR primers tested, highlighted is the primer pair used in subsequent experiments.

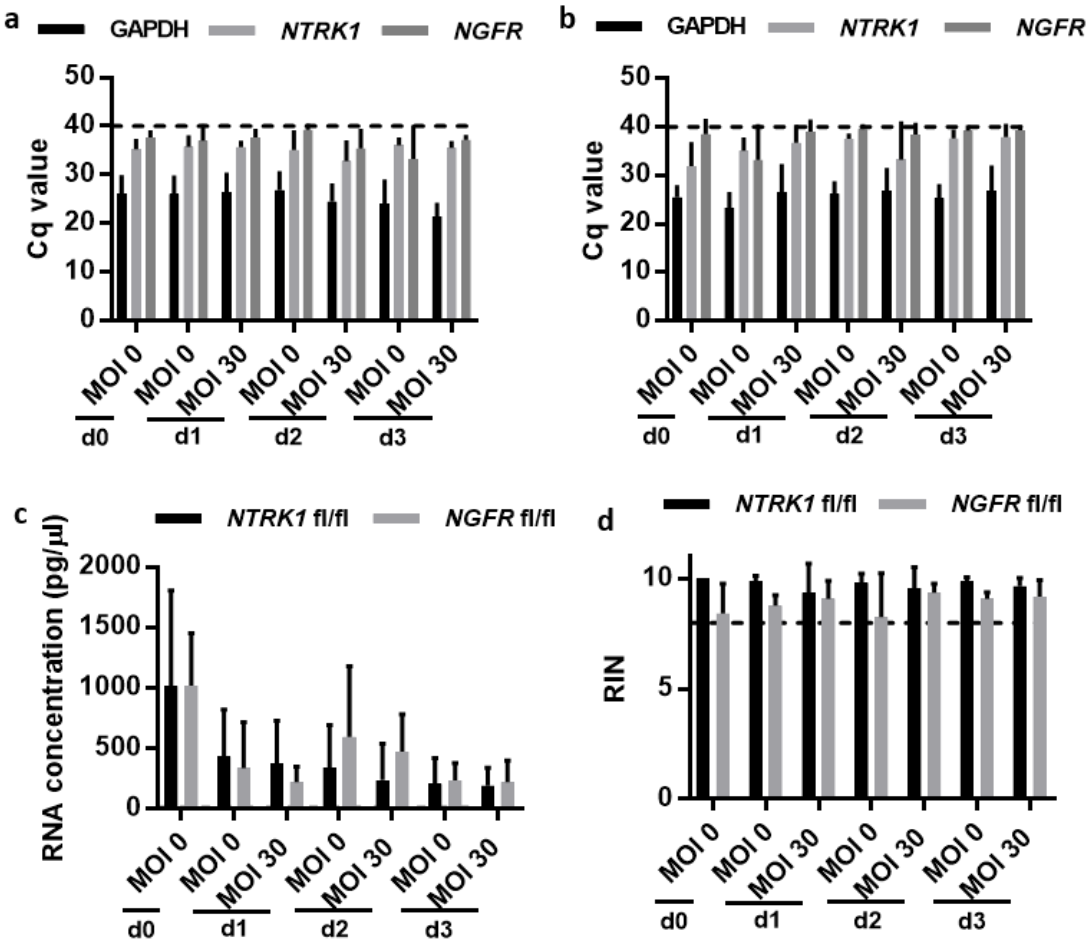


Supplementary figure 3.1 Additional neurite outgrowth measurements of MACS purified DRG neurons isolated from NTRK1 fl/fl mice treated with lenti CreGFP at MOI 30 for 3 days*. a-Longest (p=0.0172),* ***b*** *shortest and* ***c*** *mean neurite length (p=0.0360),* ***d*** *number of neurites,* ***e*** *total neurite outgrowth from longest branch and* ***f*** *overall neurite outgrowth normalised to number of neurites. One-way ANOVA with Tukey post hoc, n=2 individual cultures.*

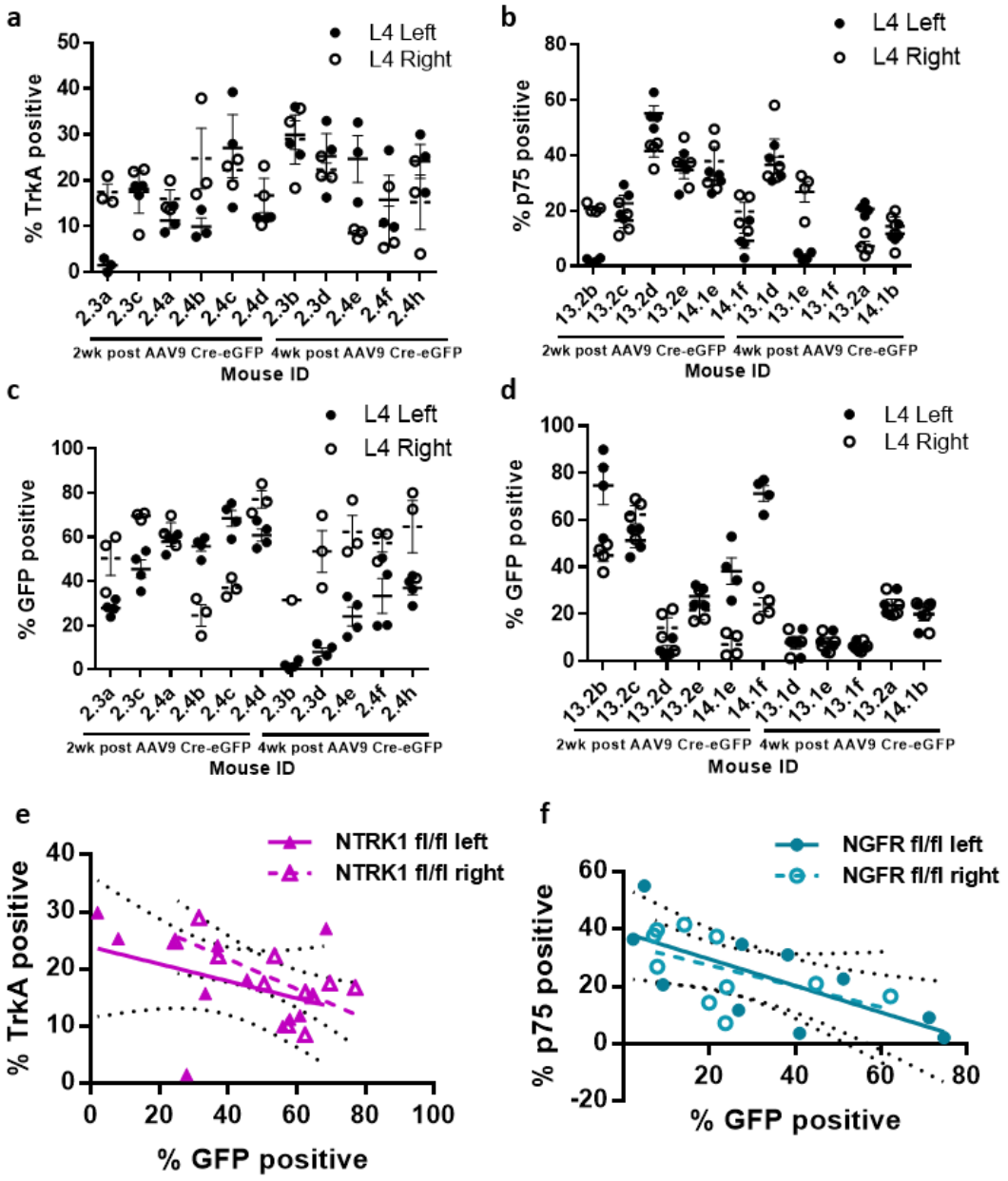


Supplementary figure 3.2 Additional neurite outgrowth measurements of MACS purified DRG neurons isolated from NGFR fl/fl mice treated with lenti CreGFP at MOI 30 for 3 days. ***a-*** *Longest,* ***b*** *shortest and* ***c*** *mean neurite length,* ***d*** *number of neurites,* ***e*** *total neurite outgrowth from longest branch and* ***f*** *overall neurite outgrowth normalised to number of neurites. One-*

way ANOVA with Tukey post hoc, neurite outgrowth measurements were not significant, n=2 individual cultures.

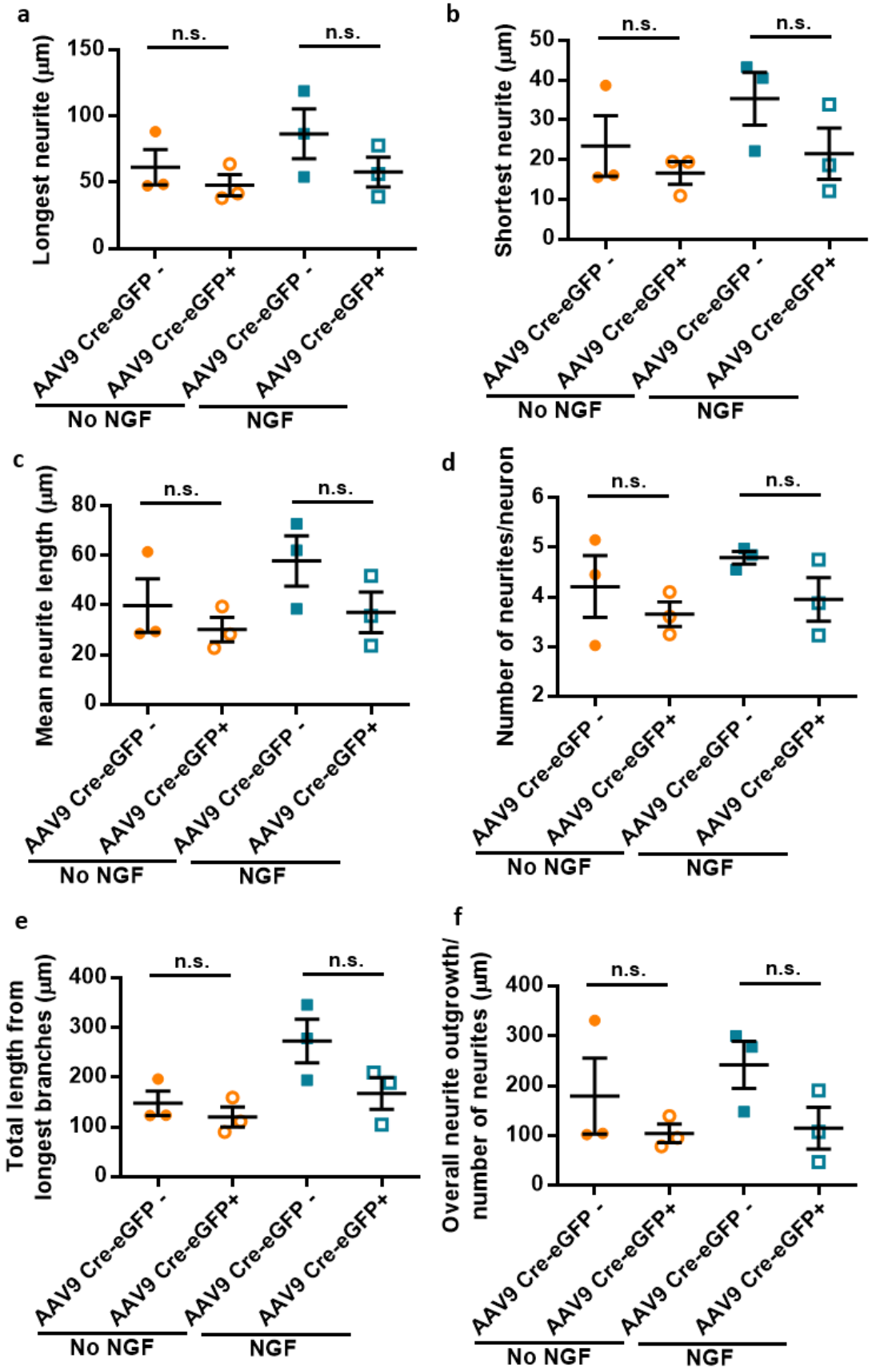


Supplementary figure 3.3 No significant difference in the expression of NTRK1 or NGFR following lenti CreGFP treatment of DRGs isolated from NTRK1 fl/fl or NGFR fl/fl mice and MACS purified. *Relative expression of GAPDH, NTRK1 and NGFR as shown by Cq values following lenti CreGFP treatment at MOI 0 or 30 for 0, 1, 2 or 3 days in vitro for* ***a*** *NTRK1 fl/fl and* ***b*** *NGFR fl/fl mice. RNA concentration* ***c*** *and RIN value* ***d*** *of RNA used. Two-way ANOVA with Tukey post-hoc showed no significant difference in gene expression, n=5 individual cultures.*

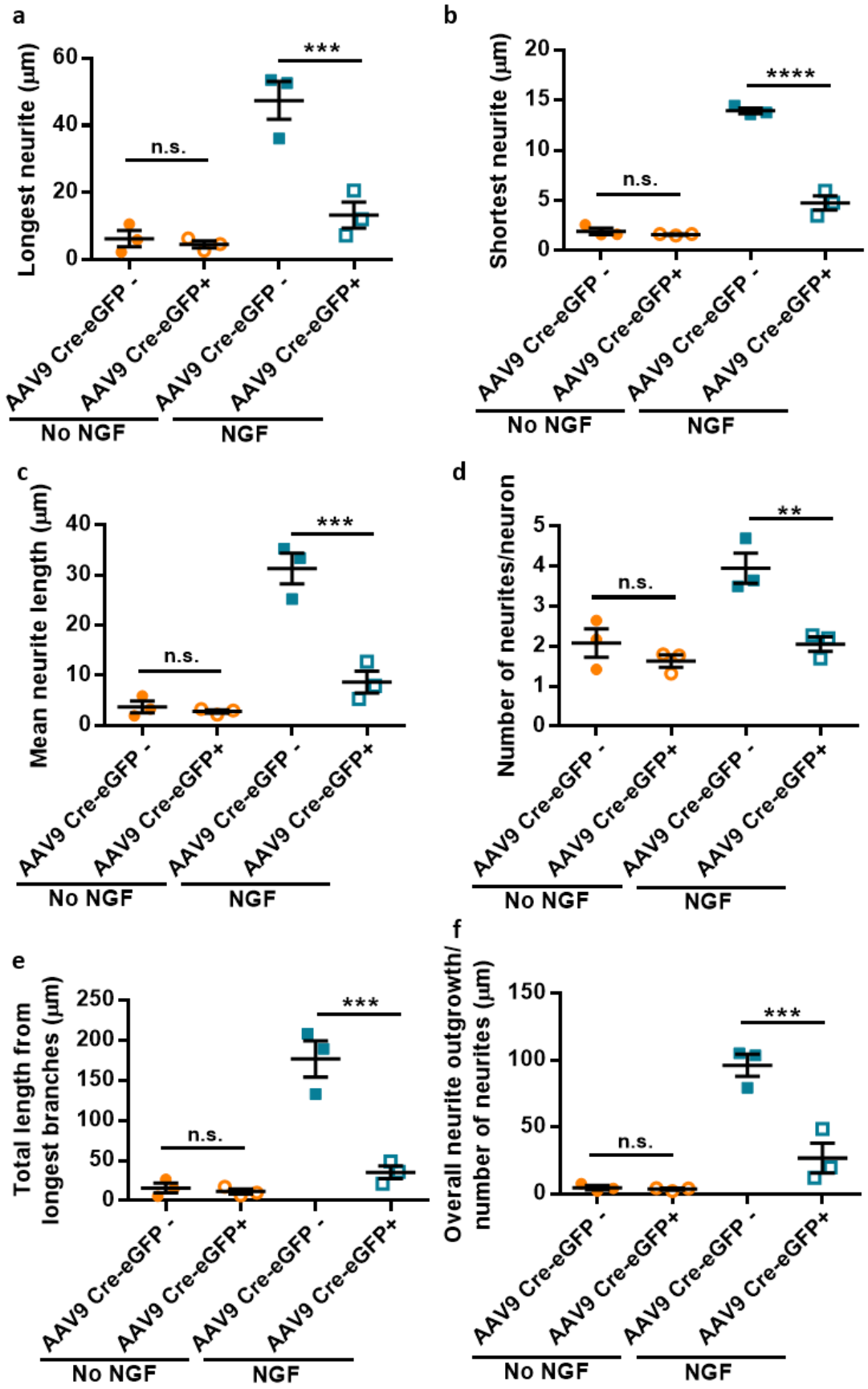


Supplementary figure 3.4 AAV9 Cre-eGFP intrathecal injections display some unilateral transduction patterns. a- *Percentage of cells expressing TrkA in the left and right L4 DRG 2 and 4 weeks post AAV9 Cre-eGFP injection in NTRK1 fl/fl mice.* ***b-*** *percentage of cells expressing p75 in the left and right L4 DRG 2 and 4 weeks post AAV9 Cre-eGFP injection in NGFR fl/fl mice.* ***c and d-*** *Percentage of cells expressing GFP in the left and right L4 DRG 2 and 4 weeks post AAV9*

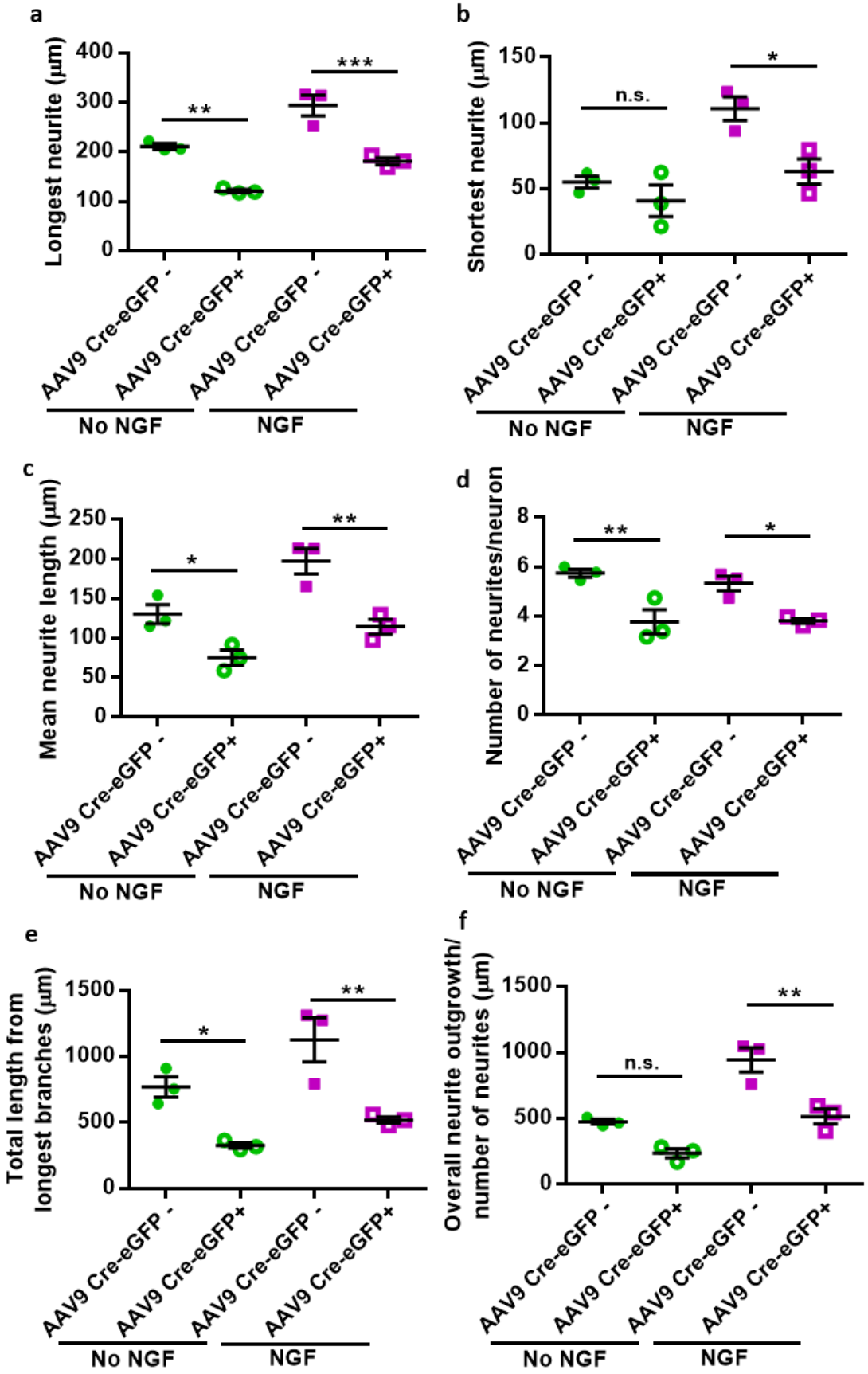
*Cre-eGFP injection in **c** NTRK1 fl/fl and **d** NGFR fl/fl mice. Correlation of GFP and TrkA or p75 expression in **e** NTRK1 fl/fl and **f** NGFR fl/fl mice respectively from left and right L4 DRG 2 and 4 weeks post AAV9 Cre-eGFP. Solid lines show linear regression and dotted lines show 95% CI. NTRK1 fl/fl left R^2=0.1355 and right R^2=0.5018. NGFR fl/fl left R^2=0.5046 and right R^2=0.2793. For all graphs n=5 animals for 2 weeks post AAV9 Cre-eGFP and n=6 for 4 weeks post AAV9 Cre-eGFP.*


a
Longest neurite (µm)
150
100
50
0
n.s.
n.s.
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF
b
Shortest neurite (µm)
50
40
30
20
10
0
n.s.
n.s.
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF
c
Mean neurite length (µm)
80
60
40
20
0
n.s.
n.s.
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF
d
Number of neurites/neuron
6
5
4
3
2
n.s.
n.s.
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF
e
Total length from longest branches (µm)
400
300
200
100
0
n.s.
n.s.
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF
f
Overall neurite outgrowth/ number of neurites (µm)
400
300
200
100
0
n.s.
n.s.
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF

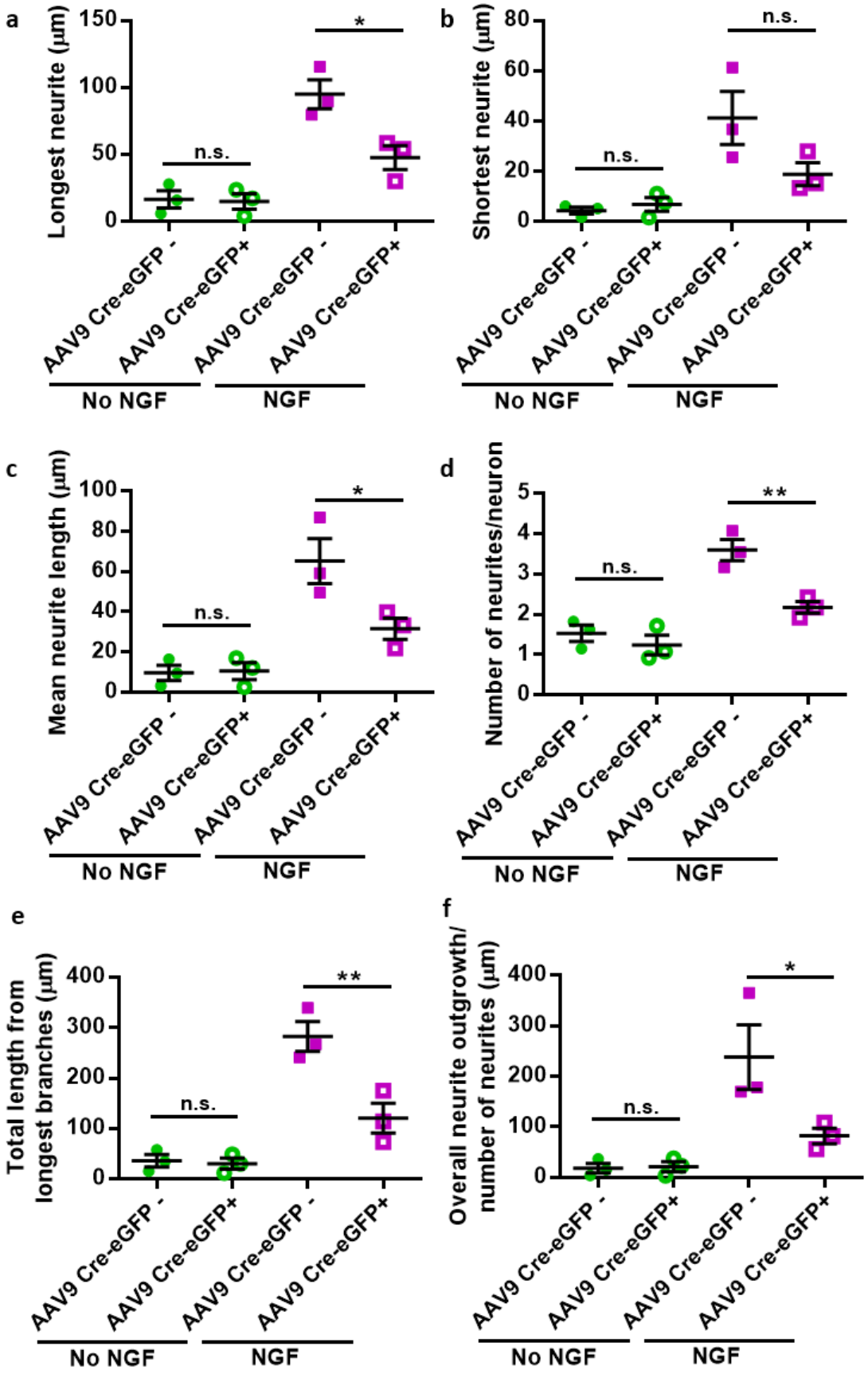
Supplementary figure 3.5 Additional neurite outgrowth measurements for non-MACS purified DRG neurons 2 weeks post intrathecal AAV9 Cre-eGFP in NTRK1 fl/fl mice. ***a-*** *Longest,* ***b*** *shortest and* ***c*** *mean neurite length,* ***d*** *number of neurites,* ***e*** *total neurite outgrowth from longest branch and* ***f*** *overall neurite outgrowth normalised to number of neurites. All neurite outgrowth parameters were not significant. One-way ANOVA with Tukey post hoc, n=3 individual cultures.*


a
Longest neurite (µm)
60
40
20
0
***
n.s.
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF
b
Shortest neurite (µm)
20
15
10
5
0
****
n.s.
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF
c
Mean neurite length (µm)
40
30
20
10
0
***
n.s.
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF
d
Number of neurites/neuron
5
4
3
2
1
0
**
n.s.
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF
e
Total length from longest branches (µm)
250
200
150
100
50
0
***
n.s.
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF
f
Overall neurite outgrowth/ number of neurites (µm)
150
100
50
0
***
n.s.
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF

Supplementary figure 3.6 Additional neurite outgrowth measurements for MACS purified DRG neurons 2 weeks post intrathecal AAV9 Cre-eGFP in NTRK1 fl/fl mice*. **a-** Longest (p=0.0008), **b** shortest (p<0.0001) and **c** mean neurite length (p=0.0002), **d** number of neurites (p=0.0068), **e** total neurite outgrowth from longest branch (p=0.0002) and **f** overall neurite outgrowth normalised to number of neurites (p=0.0005). One-way ANOVA with Tukey post hoc, n=3 individual cultures.*


a
Longest neurite (µm)
400
300
200
100
0
**
***
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF
b
Shortest neurite (µm)
150
100
50
0
n.s.
*
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF
c
Mean neurite length (µm)
250
200
150
100
50
0
*
**
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF
d
Number of neurites/neuron
8
6
4
2
0
**
*
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF
e
Total length from longest branches (µm)
1500
1000
500
0
*
**
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF
f
Overall neurite outgrowth/ number of neurites (µm)
1500
1000
500
0
n.s.
**
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF

Supplementary figure 3.7 Additional neurite outgrowth measurements for non-MACS purified DRG neurons 2 weeks post intrathecal AAV9 Cre-eGFP in NGFR fl/fl mice. ***a-*** *Longest (No NGF p=0.0026 and NGF p=0.0006),* ***b*** *shortest (p=0.0252) and* ***c*** *mean neurite length (No NGF p=0.0478 and NGF p=0.0055),* ***d*** *number of neurites (No NGF p=0.0075 and p=0.0312),* ***e*** *total neurite outgrowth from longest branch (No NGF p=0.0410 and NGF p=0.0077) and* ***f*** *overall neurite outgrowth normalised to number of neurites (p=0.0035). One-way ANOVA with Tukey post hoc, n=3 individual cultures.*


a
Longest neurite (µm)
150
100
50
0
n.s.
*
b
Shortest neurite (µm)
80
60
40
20
0
n.s.
n.s.
c
Mean neurite length (µm)
100
80
60
40
20
0
n.s.
*
d
Number of neurites/neuron
5
4
3
2
1
0
n.s.
**
e
Total length from longest branches (µm)
400
300
200
100
0
n.s.
**
f
Overall neurite outgrowth/ number of neurites (µm)
400
300
200
100
0
n.s.
*
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
AAV9 Cre-eGFP -
AAV9 Cre-eGFP+
No NGF
NGF

Supplementary figure 3.8 Additional neurite outgrowth measurements for MACS purified DRG neurons 2 weeks post intrathecal AAV9 Cre-eGFP in NGFR fl/fl mice*. **a-** Longest (p=0.0146), **b** shortest and **c** mean neurite length (p=0.0332), **d** number of neurites (p=0.0073), **e** total neurite outgrowth from longest branch (p=0.0041) and **f** overall neurite outgrowth normalised to number of neurites (p=0.0444). One-way ANOVA with Tukey post hoc, n=3 individual cultures.*

Chapter 4: Generation of a new conditional knockout mouse line for TrkA and p75

4.1 Introduction

In the previous chapter (chapter 3), we used both *NTRK1* fl/fl and *NGFR* fl/fl mouse lines in combination with viral vectors expressing Cre recombinase to induce knockout of *NTRK1* and *NGFR* genes, which encode the NGF receptors TrkA and p75, respectively. We used a lenti Cre-GFP virus *in vitro* and IT injection of an AAV9 Cre-eGFP *in vivo* to deliver Cre recombinase. However, both of these methods were found to have disadvantages. Therefore, we decided to use another approach to generate TrkA and p75 knockout. We bred these floxed lines with a mouse line encoding tamoxifen-inducible Cre recombinase under the *Advillin* promoter which is only expressed in sensory and sympathetic neurons. This allows allow both spatial and temporal control of the gene knockout in animals, thus allowing us to bypass the developmental impact of early knockout of either NGF receptor or its ligand NGF observed in global knockout mice (Chapter 1.2)(Crowley et al. 1994; Smeyne et al. 1994; K. F. Lee et al. 1992; Bergmann et al. 1997).

4.1.1 Generation of spatial-temporal controlled transgenic lines

4.1.1.1 Spatially controlled conditional knockout lines

As previously described the Cre-loxP system has been identified as an invaluable tool for using temporal and spatial control of gene expression and functional genomics (Chapter 3.1.2) (Branda and Dymecki 2004). Brown *et al.* generated knockout mouse lines using zinc-finger nucleases (ZFNs) delivered using pronuclear microinjections into fertilised rat eggs. They used 2 pairs of ZFNs targeted against 2 specific genes where each pair of ZFNs cleaved an intron flanking the region of the exon of interest and inserted a loxP site. Generation of a second transgenic mouse expressing *Cre* fused to an internal ribosomal entry site (IRES) cassette under the control of an endogenous promoter, where the IRES cassette allows separate transcription of the Cre after translation of the gene upstream of the insertion site, i.e. the promoter gene of the Cre. In this study IRES-*Cre* was injected by pronuclear injection into a fertilised egg as part of a donor plasmid in combination with a pair of ZFN which promotes insertion of the IRES-*Cre* immediately after the translational stop of the *tyrosine hydroxylase* (*th*) gene, therefore placing the Cre under the control of the t*h* promoter. Newly generated floxed and Cre mouse lines were then crossed and tissue specific knockout was observed of the floxed genes (A. J. Brown et al. 2013).

4.1.1.2 Temporally controlled conditional knockout lines

Following success using spatial restriction placing the *Cre* gene expression under the control of specific promoters, studies were performed to place the *Cre* gene under temporal control following fusion of the *Cre* gene with the hormone binding oestrogen receptor (ER). In order to prevent activation of the Cre recombinase by endogenous oestrogen Feil *et al* reported how targeted triple mutations in the ligand binding domain (LBD) of the ER resulted in ligand specific activation of the Cre recombinase and insensitivity to natural ligand 17β-oestradiol

(E2). They found that using either the G400V/M543A/L544A or G400V/L539A/L540A triple mutation by site directed recombination of the human ER fused to the Cre recombinase from bacteriophage P1 resulted in ligand specific activation from synthetic oestrogen only, such as 4-hydroxytamoxifen (OHT) and ICI 182,780 (ICI) respectively (R. Feil et al. 1997). Hayashi *et al* describes how they bred a new Cre-ER^{TM} mouse line, also insensitive to endogenous oestrogen but generating by mutating a different site in the LBD of the ER and under the control of the β-actin promoter, where Cre recombinase can be ubiquitously expressed in all cells, induced by tamoxifen. It has further been shown that Cre expression can effectively be activated with tamoxifen treatment *in utero* or postpartum or with the active metabolite of tamoxifen 4OH-TM *in vitro* (Hayashi and McMahon 2002). In parallel, Metzger *et al* generated a Cre-ER^{T} transgenic mouse line by inducing a point mutation in the LBD of ER resulting in Gly521Arg mutation. This mutated Cre was expressed under the control of the CMV promoter, which is expressed in several tissues. These mice were then crossed into a floxed RXRα mouse line and the Cre activated by tamoxifen injection. Subsequent expression as measured using RT-PCR of the affected tissues showed successful downregulation of RXRα after tamoxifen treatment (Metzger and Chambon 2001). Cre-ER^{T} mice were bred with an ACZL lacZ reporter line, which carries a CMV enhancer β-actin promoter-loxP- chloramphenicol acetyltransferase (CAT) cassette-loxP-lacZ cassette, whereby the lacZ reporter is only expressed following Cre mediated excision of the CAT cassette. It was observed that at later time points following tamoxifen injection lacZ expressing cells in the tail migrated from the granular layer into the cornified layer of the epidermis. This suggests that in this model there was no tamoxifen mediated excision of the CAT cassette in the proliferative basal keratinocytes of the epidermis as the lacZ reporter was lost over time as the epidermis was renewed. Therefore, it is important to note that for specific gene knockout of floxed genes in proliferative cells, unless

Cre recombinase is active in progenitor cells repeated treatment of tamoxifen is required (Metzger and Chambon 2001).

It has been found that the triple mutation G400V/M543A/L544A in the LBD of the ER described above in the CreERT2 mouse is three to four fold more sensitive to OHT in F9 cells compared to Cre-ER^{T}. It has therefore been hypothesised that this mutation is more potent in mice too (R. Feil et al. 1997; Metzger and Chambon 2001; Leone et al. 2003). This Cre-ERT2 containing this triple mutation in the ER has since been used in a number of studies, including neuroscientific studies by fusing Cre-ERT2 to a cell line specific promoter (S. Feil, Valtcheva, and Feil 2009). For example, a study by Leone *et al* used the Cre-ERT2 line to generate novel mouse lines for targeted gene ablation in oligodendrocytes and Schwann cells, the myelinating glial cells of the nervous system. First, they inserted CreERT2 into a proteolipid protein (PLP) regulatory region which is sufficient to direct specific transgene expression in oligodendrocytes and Schwann cells (PLP-CreERT2). They also inserted CreERT2 under the control of the P0 promoter driving the connexin32 gene to generate a Schwann cell specific inducible Cre line (P0Cx-CreERT2). Characterisation of these lines revealed that they result in cell specific knockouts across both the CNS and the PNS (Leone et al. 2003). A sensory and sympathetic neuron specific Cre mouse line using the CreERT2 has also been developed. Lau *et al* generated an *Advillin*-CreERT2 line, where *Advillin* is an actin-binding protein of the gelsolin family which is expressed in sensory and sympathetic neurons. A bacterial artificial chromosome (BAC) was used to generate this line. It contained part of exon 2 of the *Advillin* gene which contains an ATG start codon. The CreERT2 was fused to this ATG start codon of the *Advillin* gene. This BAC was then injected into embryos using pronuclear injection and offspring were screened to test the number of copies of BAC inherited. They found here that the generated mouse line contained 10 copies of the BAC transgene (Lau et al. 2011).

4.1.2 Delivery methods of tamoxifen to Cre-ER transgenic mouse lines

There have been several treatment regimens described for treatment of floxed mice with tamoxifen, a drug which is metabolised into the active metabolite OHT (S. Feil, Valtcheva, and Feil 2009). Feil *et al* 2009 summarises different methods which have been used to induce gene knockout in animals using tamoxifen. This includes *in utero* methods where pregnant females have been given i.p. doses of tamoxifen at 1 mg at E12.5, oral doses of 3-4 mg at E8.5-11.5, oral doses of 5-10 mg for up to 3 days at E12.5-13.5 or i.p. injection of OHT directly of 1 mg for 3 days at E12.5-14.5. Postnatal delivery of tamoxifen has also been shown to be successful via different routes. i.p. injection of 1mg tamoxifen for 5 consecutive days in lactating mothers where the tamoxifen is passed pups indirectly via milk from the mother or directly to the pup by i.p. injection of 0.25-1 mg tamoxifen for 5 consecutive days, oral delivery or intragastric injection of 0.05-0.1 mg for 3 consecutive days or topical skin exposure to 0.05-1 mg OHT for 3 consecutive days. Adult treatment with tamoxifen also includes a range of doses and routes. This includes 0.01-1 mg OHT or tamoxifen i.p. for 5 consecutive days, oral 1 mg tamoxifen for 5 consecutive days in 5 week old mice or 1-5 mg oral tamoxifen for 5 days in 4-8 week old mice (S. Feil, Valtcheva, and Feil 2009). Differences in treatment methods vary according to the age of mice and the target tissue. However, there is no standard method even within these parameters. There is evidence that high doses of tamoxifen *in utero* can result in failure for embryos to develop to term as has been found with Cre-ER^{TM} mice (Hayashi and McMahon 2002). Having said that, there was no difference in expression from tamoxifen injections from 0.5-3 mg although higher doses induced faster recombination (Hayashi and McMahon 2002). Kinetics of the efficiency of Cre-ER^{T} following treatment of tamoxifen was investigated by crossing Cre-ER^{T} mice this line with an ACZL reporter mouse which expressed lacZ in the presence of Cre as described above (chapter 4.1.1.2), which can be visualised using β-galactosidase staining. Mice were given i.p. injection of 1 mg tamoxifen for 5 consecutive days

and compared to controls. Expression of β-galactosidase was observed 2 to 3 days after tamoxifen treatment in the granular layer of the tail (Metzger and Chambon 2001). Crossing of the PGP-CreERT2 and P0Cx-CreERT2 oligodendrocyte and Schwann cell specific Cre mouse lines with a R26R reporter line which contains a loxP-STOP-loxP lacZ site, was used to generate a reporter line to test the specificity of the Cre line, where lacZ is transcribed following the activation of Cre. For cells within the nervous system five days of consecutive injections of 1 mg tamoxifen in 8 week old mice was found to be sufficient to achieve knockout in the lines in the CNS. However, for effective transduction of the PNS it was found that twice daily injections of 1 mg tamoxifen for 5 consecutive days was required (Leone et al. 2003). Lau *et al* also used 2 mg tamoxifen injections for 5 consecutive days to induce Cre expression in AdvCreERT2 mice crossed with a *Rosa26LacZ* reporter mouse where lacZ expression was observed in sensory neurons (Lau et al. 2011). Recently, a study has been published which for the first time investigated the toxic effects of tamoxifen in mice. In the *Atf3*-CreERT2 mouse line the CreERT2 was under the control of Atf3, a marker of cellular stress. This line was bred with a TdTomato reporter line where the presence of active Cre recombinase results in the expression of the TdTomato marker protein. This study found that tamoxifen alone resulted in expression of TdTomato in areas of the brain such as the olfactory bulb and the dentate gyrus in addition to peripheral sensory neurons. Further investigation suggests that upregulation of this stress associated marker was the result of tamoxifen mediated inhibition of cholesterol epoxide hydrolase (ChEH). However, expression was mitigated by dissolving tamoxifen in wheat germ oil which is rich in vitamin E compared to the commonly used sunflower oil, with a treatment regime of 75 mg/kg (1.5 mg) i.p for 3 consecutive days (Denk et al. 2015).

4.1.3 Aim of the study

The aim of this part of the study was to generate a knockout mouse line that allows spatial and temporal control of either the TrkA or the p75 NGF receptor. By crossing *NTRK1* fl/fl and *NGFR*

fl/fl mouse lines with an AdvCreERT2 (Lau et al. 2011) line we aimed to generate conditional inducible knockout lines for both NGF receptors. Conditional knockout of the *NTRK1* and *NGFR* genes will be restricted to cells expressing the *Advillin* gene by using a Cre mouse line that expresses Cre under the control of the *Advillin* promoter. Since the *Advillin* gene is expressed in sensory and sympathetic neurons there is spatial control of gene knockout. Fusion of Cre recombinase to a mutated ER (ERT2) results in the Cre recombinase remaining inactive and bound to heat shock protein 90 (HSP90) except in the presence of tamoxifen, where binding of tamoxifen to the LBD results in confirmation change of the Cre protein and the HSP90 is released and active Cre then moves to the nucleus where it recognises and cleaves LoxP sites (Leone et al. 2003). This therefore allows temporal control of gene knockout. Once the *NTRK1* fl/fl; AdvCreERT2 and *NGFR* fl/fl; AdvCreERT2 mouse lines were generated the second aim of this part of the study was to use tamoxifen (TMX) to induce knockout of the *NTRK1* and *NGFR* genes respectively in Cre positive mice, characterise the effectiveness of the knockout at the RNA and protein level, and investigate the adult phenotype following gene knockout.

4.2 Methods

4.2.1 Animals

All animals were housed in a designated facility and maintained in accordance with ASPA and Home Office regulations. Animals were kept in a 12hr light dark cycle and fed *ad libitum*. All animals were sacrificed using schedule 1. *NTRK1* fl/fl ($Ntrk1^{tm1c(EUCOMM)Wtsi}$) and *NGFR* fl/fl ($Ngfr^{tm1c(EUCOMM)Wtsi}$) transgenic mice obtained from the International Mouse Phenotype Consortium (IMPC) from Harwell, previously described in chapter 3.1.1 were crossed with *Advillin* CreERT2 (AdvCreERT2) transgenic mice (kindly provided by Prof John Wood, UCL). AdvCreERT2 mice were backcrossed into the C57Bl6 background for 5 generations prior to crossing to the other transgenic lines. It is important to note that as AdvCreERT2 mouse is

generated using pronuclear injection of a BAC containing the fused *Advillin* and CreERT2 gene where there is insertion of the BAC into the genome at several sites and therefore these mice are hemizygous and defined as either Cre negative or Cre positive based on genotype. There is no homozygous or heterozygous AdvCreERT2. Homozygous *NTRK1* fl/fl and *NGFR* fl/fl mice were crossed with hemizygous AdvCreERT2 mice. The resulting heterozygous *NTRK1* +/fl or *NGFR* +/fl AdvCreERT2 positive mice were then crossed in order to generate homozygous *NTRK1* fl/fl or *NGFR* fl/fl; AdvCreERT2 positive mice respectively. All animals were sacrificed according to Schedule 1 procedure.

4.2.2 Genotyping

As described in the methods section of chapter 3.2.2.

Progeny from the crossings of *NTRK1* fl/fl and *NGFR* fl/fl mice with AdvCreERT2 were genotyped using the specific primers for the floxed allele of *NTRK1* or *NGFR* (as described in chapter 3.2.2) in addition to two sets of primers for the AdvCreERT2 inheritance in separate PCR reactions. For the AdvCre primers, WT bands were observed at 480 bp and mutant bands at 180 bp. For the CreERT2 primers a mutant band was observed at 435 bp.

The AdvCreERT2 founder colony was also crossed with another floxed line *LIFR* fl/fl ($Lifr^{tm1c(EUCOMM)Hmgu}$) from the International Mouse Phenotype Consortium (IMPC) from Harwell, details of the number of mice FD and inheritance of the Cre gene are included in **Fig 4.2**. Similar to the *NTRK1* fl/fl and *NGFR* fl/fl lines *LIFR* fl/fl mice contain a loxP flanked region of a critical region of the gene for the lif receptor which can be excised and expression knocked out through action of Cre recombinase.

*NTRK1*_F: GCAGTATTGCAGGTGTCCCA

*NTRK1*_R: AGGACCAAGGCTCTCACAAC

*NGFR*_F: AGACACCTCCAGTACAGAGTCAA

*NGFR*_R: CCCTTCTCCAGGTCTCATTCC

*LIFR*_F: GCTCTGCTCCTGGAAGACAC

*LIFR*_R: GTGGCATGATAATAAACCGTTGAGA

*AdvCre*_F: CCCTGTTCACTGTGAGTAGG

*AdvCre*_WT_R: AGTATCTGGTAGGTGCTTCCAG

*AdvCre*_Mut_R: GCGATCCCTGAACATGTCCATC

*CreERT2*_F: CACCGCCTGATCTATGGTGC

*CreERT2*_R: TGCCAGGTTGGTCAGTAAGC

4.2.3 Tamoxifen dosing

Tamoxifen was prepared as follows: 0.195g of tamoxifen (Sigma, T5648) was dissolved in 2ml of 100% ethanol. Solution was vortexed until the tamoxifen was dissolved and diluted in 23ml of wheat germ oil resulting in a final tamoxifen concentration of 7.8 µg/µl. The wheat germ oil was placed on a shaker at RT and left until it was well mixed, typically 15-20 min. It was then aliquoted and stored at 4^{o}C for up to 3 days or stored at -20^{o}C for up to 3 months. Adult mice 9-12 and 19 week old were intraperitoneally (i.p.) injected with tamoxifen at 75 mg/kg i.p. for 3 consecutive days.

4.2.4 Immunohistochemistry

As described in the methods section of chapter 3.2.8.

Using the cryostat sections of lumbar spinal cord around the L3 to L5 region were cut to a thickness of 20 µm and sections of plantar skin was cut to a thickness of 10 µm. Skin from both

hind-paws was collected. However, in this study we only measured the number of intraepidermal nerve fibres (IENF) in the left paw for each animal. Contrary to DRG and spinal cord sections, plantar skin was also collected onto gelatine coated slides. Briefly, gelatine coated slides were prepared by distilled water by dissolving 1.5g of Gelatine type A (Sigma, G2500) and 0.25g chromium potassium sulphate (Sigma, 34636) in 500 ml distilled water heated to 60^{o}C. The solution was then cooled to 40-50^{o}C and racks of slides dipped twice to coat. Slides were then covered and left in a dust free environment overnight to dry before use.

The following primary antibodies were used for this chapter: 1:200 anti-TrkA goat polyclonal (R&D systems, AF1056), 1:1000 anti-β-III tubulin rabbit polyclonal (Abcam, ab18207), 1:500 anti-p75 NGF receptor rabbit polyclonal (Abcam, ab8875), 1:500 anti-PGP 9.5 rabbit polyclonal (Ultraclone, RA95101) and 1:1000 anti-NeuN rabbit mAb (cell signalling, 12943).

The following secondary antibodies were used for this chapter: 1:1000 Chicken anti-goat IgG (H+L) Alexa Fluor® 488 (ThermoFisher, A21467), 1:1000 donkey anti-rabbit IgG (H+L) Alexa Fluor® 488 (ThermoFisher, A21206), 1:1000 donkey anti-rabbit IgG (H+L) Alexa Fluor® 568 (ThermoFisher, A10042) and 1:1000 donkey anti-mouse IgG (H+L) Alexa Fluor® 488 (ThermoFisher, A21202).

4.2.5 RT-PCR

As described in the methods section of chapter 3.2.11.

4.2.6 RNA sequencing

L2, 3 and 5 DRGs from both sides of the spinal cord were collected from *NTRK1* fl/fl littermate controls and *NTRK1* fl/fl; AdvCreERT2 mice 4 weeks after i.p. injection with tamoxifen. At the time of injection mice were 12-19 weeks of age. DRGs were triturated and MACS purified as described in the methods section of chapter 2 (chapter 2.2.3). Following isolation, MACS

purified neurons were lysed and RNA was isolated and purified as described in the methods section of chapter 3 (chapter 3.2.9) except that the quantity and quality of RNA was measured using the Agilent RNA 6000 pico kit (Agilent technologies, 5067-1535) and measured using the Agilent2100 Bioanalyzer (Agilent, Santa Clara, CA). Once confirmed that there was sufficient RNA and the RIN for each sample was above 8, samples were transferred to a 96 well plate and sent off to high throughput genomics at the Oxford genomics centre, University of Oxford, for RNA sequencing. Samples were run on an Illumina HiSeq4000 platform. Analysis was performed using the online bioinformatics software the galaxy project with the Tuxedo suit. Files were re-aligned and mapped back to the genome using TopHat2. Fragments per kilobase per million (FPKM) was then estimated using Cufflinks and then the differential expression measured using CuffDiff (Thakur et al. 2014; Michalovova et al. 2015). Data was then sorted according to unadjusted p value with a first cut-off of $p \leq 0.0001$. When the data was split into males and females it was observed that there was a larger number of differentially regulated genes in males alone compared to males and females combined. However, with the first cut-off for p values in the males only *NTRK1* expression was not included in the differentially regulated genes between WT and CKO animals. Applying a second cut-off of $p \leq 0.001$ included the *NTRK1* gene in the list of genes differentially regulated (total 184 genes). This second cut-off was also applied to data for males and females combined and a list of 63 differentially expressed genes obtained. Data was then sorted according to log2 fold change to arrange data based on the number of genes up and down regulated. Heat maps were plotted for differentially regulated genes for males and females combined and males only using FPKM values.

4.2.7 Behavioural tests

Thermal hypersensitivity was measured using the static hot plate test (Hunskaar, Berge, and Hole 1986). A hot plate was set to a constant temperature of 52^{o}C and mice were placed onto

the plate, and a timer started. The time taken for the mouse to respond as seen by a flicking, licking or biting behaviour of the hind paw was measured. We used a 40 second cut-off where any animal that failed to respond within 40 seconds was removed from the hot plate to prevent injury. This test was performed at either 2 or 4 weeks post the first tamoxifen injection for two separate cohorts of *NTRK1* fl/fl mice where for the 2 week cohort 4 control and 4 knockout animals were used and for the 4 week cohort 7 control and 7 knockout animals were used. For *NGFR* fl/fl animals a single cohort of animals was tested on the static hot plate after 2 weeks where 4 control animals and 3 knockout animals were used.

Mechanical hypersensitivity was measured using Von Frey hairs using the SUDO up down method (Bonin, Bories, and De Koninck 2014). Briefly, mice were introduced to the test boxes and acclimatised for 45 minutes to acclimatise prior to testing. All mice were first tested on the left hind paw with the 0.6g Von Frey hair. Hairs were placed into the centre of the paw twice to assure the response was accurate. A positive response to the hair was defined as a flicking or licking of the hind paw. In case of a positive response the subsequent hair used for testing was a lower weight, for non-responders the weight of the Von Frey hair was increased by one level for the subsequent round of testing. Once all animals were tested with the first hair on the left paw, the right paw was tested with the first hair before proceeding to the left paw for the second hair. The testing alternated between the left and right hind paws for all animals until 5 responses had been measured for each paw. All animals were given 3 baseline measurements over 3 subsequent days 1 week prior to tamoxifen treatment. The test was then repeated 1, 2, 3 and 4 weeks post the first tamoxifen injection. The 50% threshold for withdrawal was then calculated for each paw for all animals (Chaplan, SR, Bach, FW, Pogrel, JW, Chung, JM, Yaksh 1994).

4.2.8 Imaging and analysis

Images of immunohistochemistry were obtained as described in the methods section of chapter 2.2.7. A minimum of 3 sections of a L4 DRG or lumbar spinal cord was imaged per animal.

Expression of TrkA or p75 in the L4 DRG was measured using the cell counter plugin of Fiji. The cell size distribution was measured by circling the cell soma and calculating the Feret diameter from the area using Fiji.

Expression of TrkA in the spinal cord was measured by circling the area of both superficial dorsal horns in control and conditional knockout animals. The relative fluorescence was measured, normalised to the area circled and an average calculated for both superficial dorsal horns per section. Within each section a similar sized region of both ventral horns was circled and fluorescence was normalised to that area and an average background level of fluorescence was obtained from both ventral horns. The expression of TrkA in the dorsal horn as measured by the fluorescent signal was then normalised to the corresponding ventral horn control for each section. An average level of fluorescence was calculated from the 3 sections of each of the 4 control animals imaged and all data was normalised to this average to calculate the FC in expression in the conditional knockout animals.

Intraepidermal nerve fibre (IENF) density was calculated by counting the fibres staining positive for PGP 9.5, a marker for axons and small nerve fibres in peripheral tissue. The number of PGP 9.5 positive fibres in the intraepidermal space was counted from 5 sections of left hind paw plantar skin per animal. Fibres that branched prior to crossing into the intraepidermal space were counted as separate fibres, however all branches inside the intraepidermal space were counted as one fibre. The number of fibres was then normalised to the length of the skin section (mm), as measured in Fiji.

FC was calculated using the delta delta CT value normalised to *GAPDH* for RT-PCR.

4.2.9 Statistics

Graphs were plotted and statistical tests performed using GraphPad Prism 7 software.

For immunohistochemistry, expression of TrkA in the L4 DRG of *NTRK1* fl/fl; AdvCreERT2 mice was tested using a one-way ANOVA with Sidak's multiple comparison test. However, for testing a potential change in TrkA expression in the spinal cord or p75 expression in L4 DRGs in the *NGFR* fl/fl; AdvCreERT2 mice a two-tailed unpaired student t-test was used. Changes in cell size distribution in both the *NTRK1* and the *NGFR* fl/fl; AdvCreERT2 was measured using a two-way ANOVA with Sidak's multiple comparison test. Finally, for measuring changes in the IENF, a one-way ANOVA with Tukey multiple comparison test was used for *NTRK1* fl/fl; AdvCreERT2 mice and a two-tailed unpaired student t-test for *NGFR* fl/fl; AdvCreERT2 mice.

Two-tailed unpaired student t-tests were also used to measure any significant differences in gene expression from all RT-PCR experiments and changes in thermal hypersensitivity using the static hot plate. Two-way ANOVA with Tukey post-hoc test was used to assess changes in mechanical hypersensitivity using Von Frey hairs.

4.3 Results

4.3.1 Breeding a new line of conditional TrkA or p75 knockout animals

In order to investigate the role of TrkA and p75 *in vivo* we bred inducible conditional knockout mice for TrkA (*NTRK1*) and p75 (*NGFR*) KO. We crossed *NTRK1* fl/fl and *NGFR* fl/fl mice with AdvCreERT2 transgenic mice to obtain homozygous *NTRK1* fl/fl or *NGFR* fl/fl and AdvCreERT2 positive animals (**Fig 4.1**). In our experiments, we used littermate controls that were homozygous for *NTRK1* fl/fl or *NGFR* fl/fl and Cre negative.

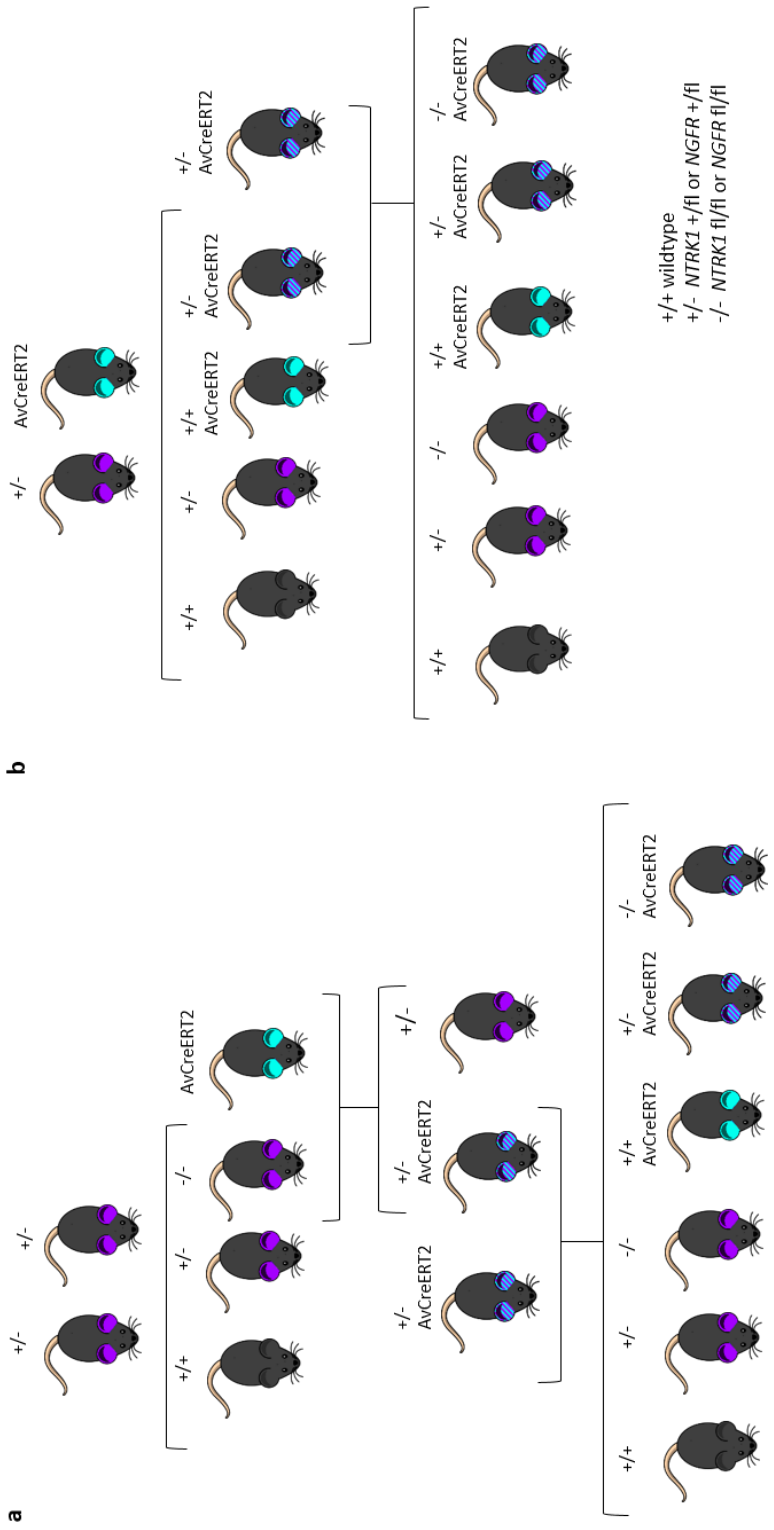


Figure 4.1 Breeding plan of transgenic mice. *Examples of the breeding plans for crossing the NTRK1 fl/fl or the NGFR fl/fl mice with hemizygous AdvCreERT2 from either a* ***a*** *homozygous or* ***b*** *heterozygous population of floxed animals.*

The breeding of these various lines proved to be problematic. A number of AdvCreERT2 positive mice were found dead (FD) for unknown reasons. Even more animals were FD when carrying a floxed allele in addition to the gene for Cre recombinase. The following percentages of Cre positive or negative mice FD from the total number of Cre positive or negative animals in the colony have been identified. For the AdvCreERT (A1645) 13.2% of animals FD were positive and 1.2% negative, for the *LIFR* fl/fl; AdvCreERT2 (A1831) 41.2% were positive and 2.4% negative, for the *NGFR* fl/fl; AdvCreERT2 (A1832) 88.9% were positive and 3.8% negative and finally for *NTRK1* fl/fl; AdvCreERT2 (A1833) 76.2% were positive and 6.1% negative. The total percentage of mice FD from the total number of mice in the colony however was not found to be high. For the AdvCreERT2 5% of animals were FD, 10.8% from the *LIFR* fl/fl; AdvCreERT2, 16.4% from the *NGFR* fl/fl; AdvCreERT2 and 13.6% from *NTRK1* fl/fl; AdvCreERT2, data taken up until the end of July 2016 (**Fig 4.2a**). It should be noted that *LIFR* fl/fl; AdvCreERT2 which are conditional knockout for the lif receptor are not used directly in this study but they were an additional colony crossed with the AdvCreERT2 line under my care and exhibited similar sign of breeding issues. Therefore, I have incorporate breeding data from this line into this study.

We then investigated if there were any environmental factors that had an impact on sudden death of animals in a given month. When split into months, it became apparent that there was a peak in death occurrence between November 2015 and February 2016, and then again between March and May 2016 in the *NGFR* fl/fl; AdvCreERT2 and *NTRK1* fl/fl; AdvCreERT2 colonies particularly (**Fig 4.2b**). Investigating the age of death revealed a wide age range at the time of death. The average age of male mice FD from the AdvCreERT2 line was 152 ± 98 days, from the *LIFR* fl/fl; AdvCreERT2 line 168 ± 68 days for males and 85 ± 39 days for females, from the *NGFR* fl/fl; AdvCreERT2 line 117 ± 74 days for males and 129 ± 64 for females and from the

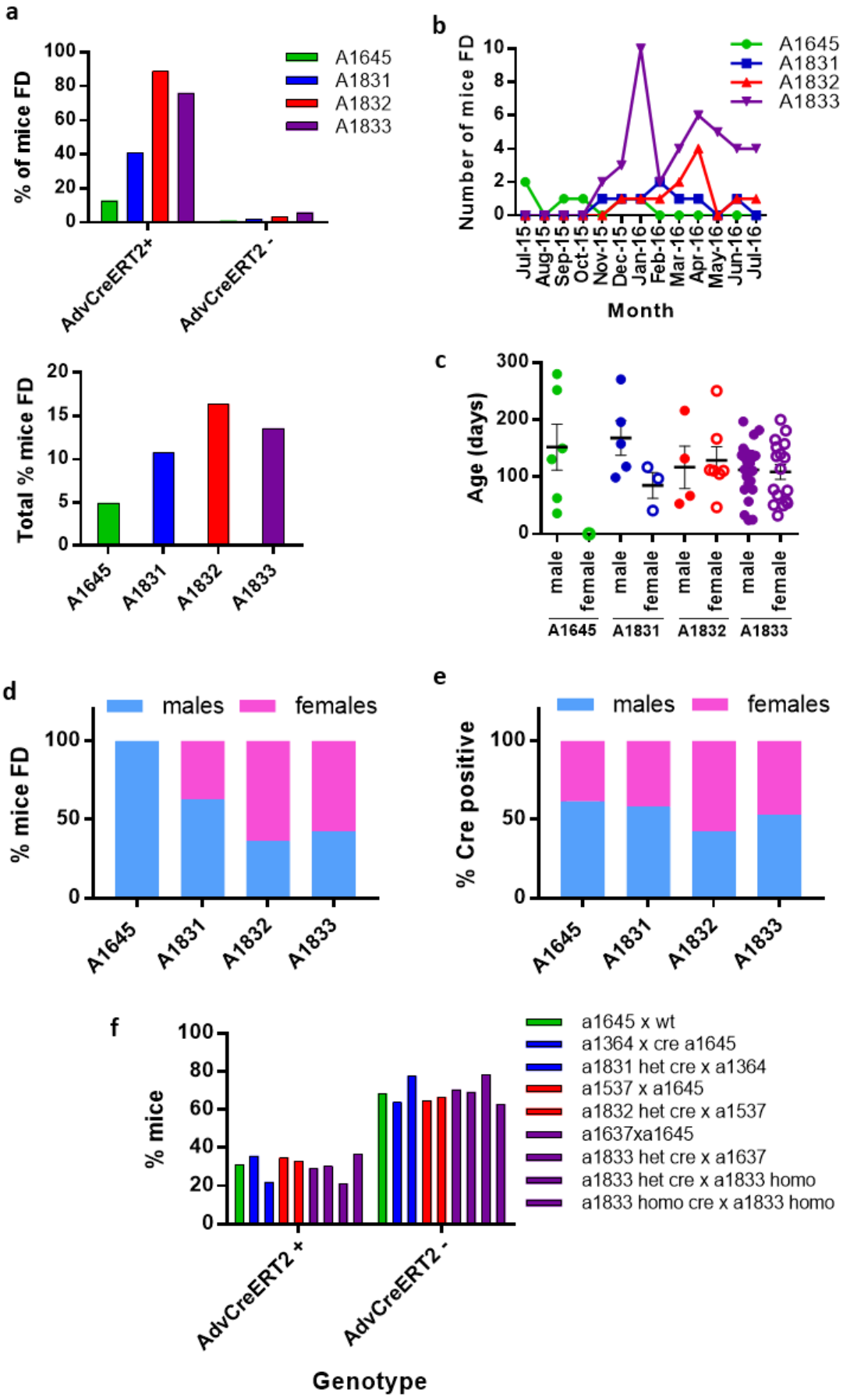

a
% of mice FD
A1645
A1831
A1832
A1833
AdvCreERT2+
AdvCreERT2 -
b
Number of mice FD
Jul-15
Aug-15
Sep-15
Oct-15
Nov-15
Dec-15
Jan-16
Feb-16
Mar-16
Apr-16
May-16
Jun-16
Jul-16
Month
Total % mice FD
c
Age (days)
male
female
d
males
females
% mice FD
e
% Cre positive
f
% mice
a1645 x wt
a1364 x cre a1645
a1831 het cre x a1364
a1537 x a1645
a1832 het cre x a1537
a1637xa1645
a1833 het cre x a1637
a1833 het cre x a1833 homo
a1833 homo cre x a1833 homo
Genotype

Figure 4.2 Breeding of transgenic animals was slow and there unexplained deaths a- *Percentage and number of mice which were found dead (FD) across various lines. These include A1645 AdvCreERT2 founder line (green), A1831 LIFR fl/fl; AdvCreERT2 cross (blue), A1832 NGFR fl/fl; AdvCreERT2 cross (red) and A1833 NTRK1 fl/fl; AdvCreERT2 cross (purple).* ***b-*** *Number of mice that were FD split into the month of death.* ***c-*** *Ages of the mice FD for each of the various transgenic mouse lines separated into males and females.* ***d-*** *Percentage of mice FD split into males and females.* ***e-*** *Percentage of mice inheriting the AdvCreERT2 gene split into males and females.* ***f-*** *Inheritance of the AdvCreERT2 across various transgenic animals (average 30.7% ± 5.6%) from different breeding strategies. All this data collected from July 2015 until July 2016.*

NTRK1 fl/fl; AdvCreERT2 line 112 ± 48 for males and 109 ± 53 days for females (**Fig 4.2c**). To evaluate if there is a gender difference, the percentage of male and female mice FD was calculated for each of the transgenic lines. We found that for the AdvCreERT2 line 100% of the mice FD were males, from the *LIFR* fl/fl; AdvCreERT2 62.5% were male and 37.5% female, from the *NGFR* fl/fl; AdvCreERT2 line 36.4% were male and 63.6% female and from the *NTRK1* fl/fl; AdvCreERT2 42.5% were male and 57.5% female (**Fig 4.2d**).

Genotyping for the Cre recombinase gene of new born litters revealed that the distribution was not occurring according to Mendelian law. We then investigated if the Cre gene inheritance had a gender preference. We therefore calculated the percentage of males and females inheriting the AdvCreERT2 gene across the four transgenic colonies. Of the number of mice having the Cre gene from the AdvCreERT2 line 61.5% were male and 38.5% female, from the *LIFR* fl/fl; AdvCreERT2 line 57.9% were male and 42.1% female, from the *NGFR* fl/fl; AdvCreERT2 line 42.1% were male and 57.9% female and lastly from the *NTRK1* fl/fl; AdvCreERT2 line 52.9% were male and 47.1% female (**Fig 4.2e**). Finally, we calculated the percentage of mice from each of the four colonies inheriting the Cre gene from the total

number of mice and found that across each of the lines – irrespective of the genotypes of the parents- there was on average 30.7% ± 5.6% of the progeny to be Cre positive (**Fig 4.2f**).

4.3.2 Conditional KO of TrkA *in vivo* results in a decrease in RNA transcript and protein expression

In order to KO TrkA *in vivo*, adult *NTRK1* fl/fl; AdvCreERT2 and *NTRK1* fl/fl littermates were injected with 75mg/kg tamoxifen (TMX) for 3 days consecutively and tissue was collected 2 or 4 weeks post the first injection. We first confirmed the KO of TrkA by measuring the level of TrkA expression in the L4 DRG.

From experience in the group it is known that TrkA antibodies need very specific conditions to be efficient so we tested different antibodies *in vitro* and in wildtype tissue first before moving into tissue from transgenic mice. A range of different antibody concentrations and fixing methods were trialled until an optimal staining protocol and antibody was found (**Supplementary Table 4.1**).

We decided to use the R&D antibody and stained tissue from *NTRK1* fl/fl and NTRK1 fl/fl; AdvCreERT2 animals given i.p. injection of TMX at 9-19 weeks of age where TMX mediated activation of *Advillin* Cre in *NTRK1* fl/fl; AdvCreERT2 animals knockout TrkA expression in sensory neurons. Both L4 DRG was collected and used for immunohistochemistry and both L3 DRG collected for RT-PCR 2 or 4 weeks after injection with TMX. Sections of lumbar cord were also collected from animals 2 weeks after TMX injection. Compared to *NTRK1* fl/fl littermates a significant decrease in TrkA protein levels was observed in *NTRK1* fl/fl; AdvCreERT2 animals both 2 ($p=0.0017$) and 4 ($p<0.0001$) weeks post-TMX, one-way ANOVA with Sidak's post-hoc test. At 2 weeks post TMX there was a decrease from 19.8% ± 11.9% in *NTRK1* fl/fl to 0.6% ± 0.4% TrkA expression in L4 DRG in *NTRK1* fl/fl; AdvCreERT2 mice. At 4 weeks post TMX there was a decrease from 33.0% ± 6.2% in *NTRK1* fl/fl to 1.8% ± 0.9% TrkA expression in L4 DRG in

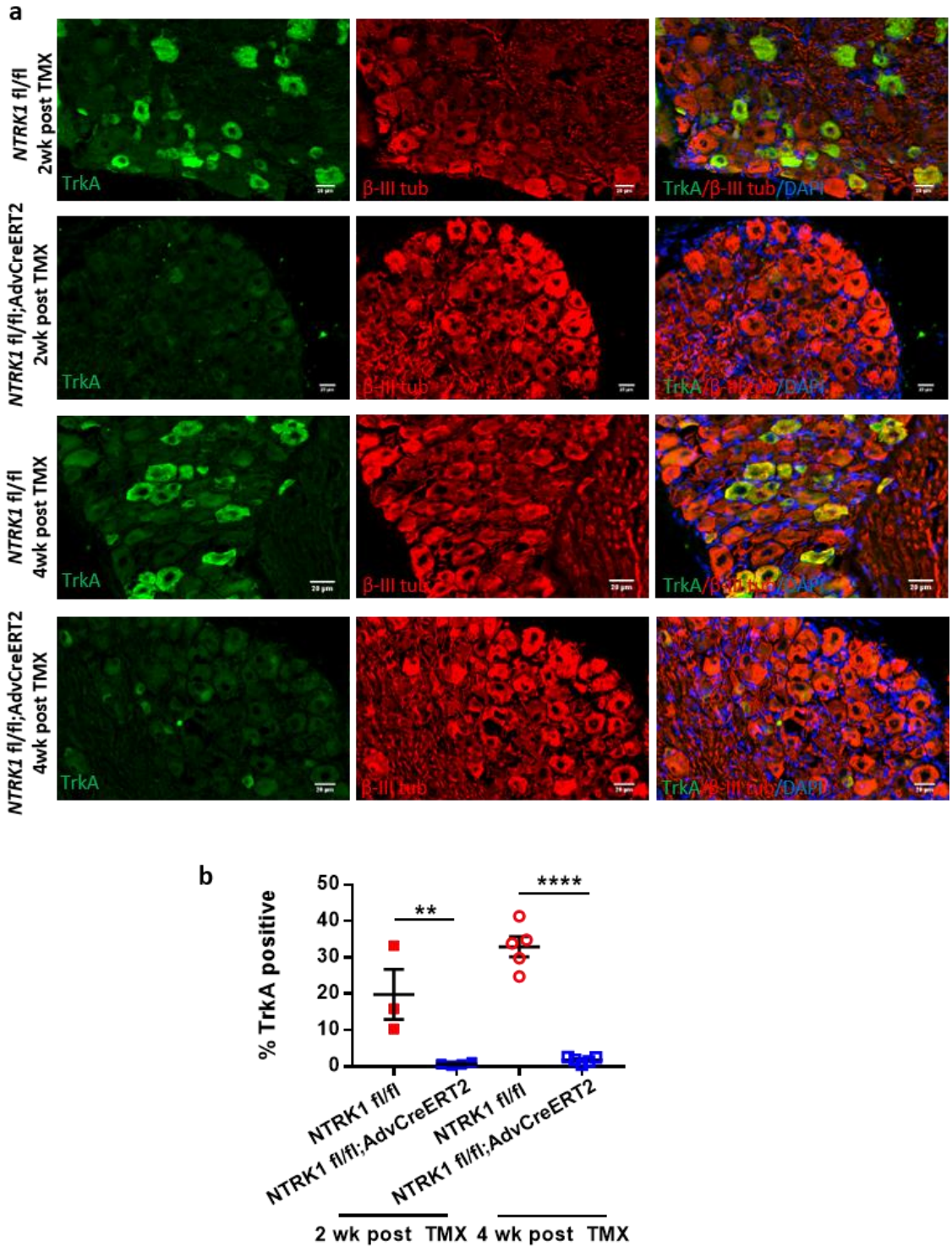


Figure 4.3 Expression of TrkA was reduced in NTRK1 fl/fl; AdvCreERT2 mice 2 and 4 weeks post tamoxifen injection. a- *Representative immunohistochemistry of TrkA expression in an L4 DRG of NTRK1 fl/fl control and NTRK1 fl/fl; AdvCreERT2 mice 2 and 4 weeks post IP injection of*

TMX. ***b****- Quantification of TrkA expression in a L4 DRG in NTRK1 fl/fl and NTRK1 fl/fl; AdvCreERT2 CKO mice 2 and 4 weeks post IP TMX (2wks p=0.0017 and 4 weeks p<0.0001). One-way ANOVA with Sidak's post-hoc, n=3-5 animals.*

NTRK1 fl/fl; AdvCreERT2 mice (**Fig 4.3**). RT-PCR on whole L3 DRG from *NTRK1* fl/fl; AdvCreERT2 mice also showed a significant decrease in *NTRK1* mRNA expression normalised to *GAPDH* compared to *NTRK1* fl/fl littermate both 2 (p=0.0006) and 4 (p=0.0456) weeks post TMX, two-tailed unpaired student t-test. There was a decrease from 1.02 ± 0.25 in *NTRK1* fl/fl to 0.17 ± 0.06 fold change at 2 weeks post TMX in *NTRK1* fl/fl; AdvCreERT2 mice and a decrease from 1.11 ± 0.49 in *NTRK1* fl/fl to 0.5 ± 0.31 fold change at 4 weeks post TMX in *NTRK1* fl/fl; AdvCreERT2 mice. Next, we investigated if the knockout of *NTRK1* affects the expression level of *NGFR*. There was no significant difference in *NGFR* expression normalised to *GAPDH* 2 weeks (1.01 ± 0.11 in *NTRK1* fl/fl to 0.93 ± 0.48 in *NTRK1* fl/fl; AdvCreERT2 mice) and 4 weeks (1.14 ± 0.65 in *NTRK1* fl/fl to 1.40 ± 0.72 in *NTRK1* fl/fl; AdvCreERT2 mice) post TMX injection (**Fig 4.4a**).

We measured TrkA expression in the superficial lamina of the dorsal horn of the spinal cord to investigate if knockout of *NTRK1* in sensory neurons correlated to reduced TrkA expression in sensory neuron terminals projecting into the cord. We observed a significant decrease in the expression of TrkA based on fluorescence intensity normalised to the background staining of the ventral cord from 1.05 ± 0.16 in *NTRK1* fl/fll littermates to 0.92 ± 0.10 in *NTRK1* fl/fl; AdvCreERT2 mice 2 weeks post TMX, p=0.0228 two-tailed unpaired student t-test (**Fig 4.4b and c**).

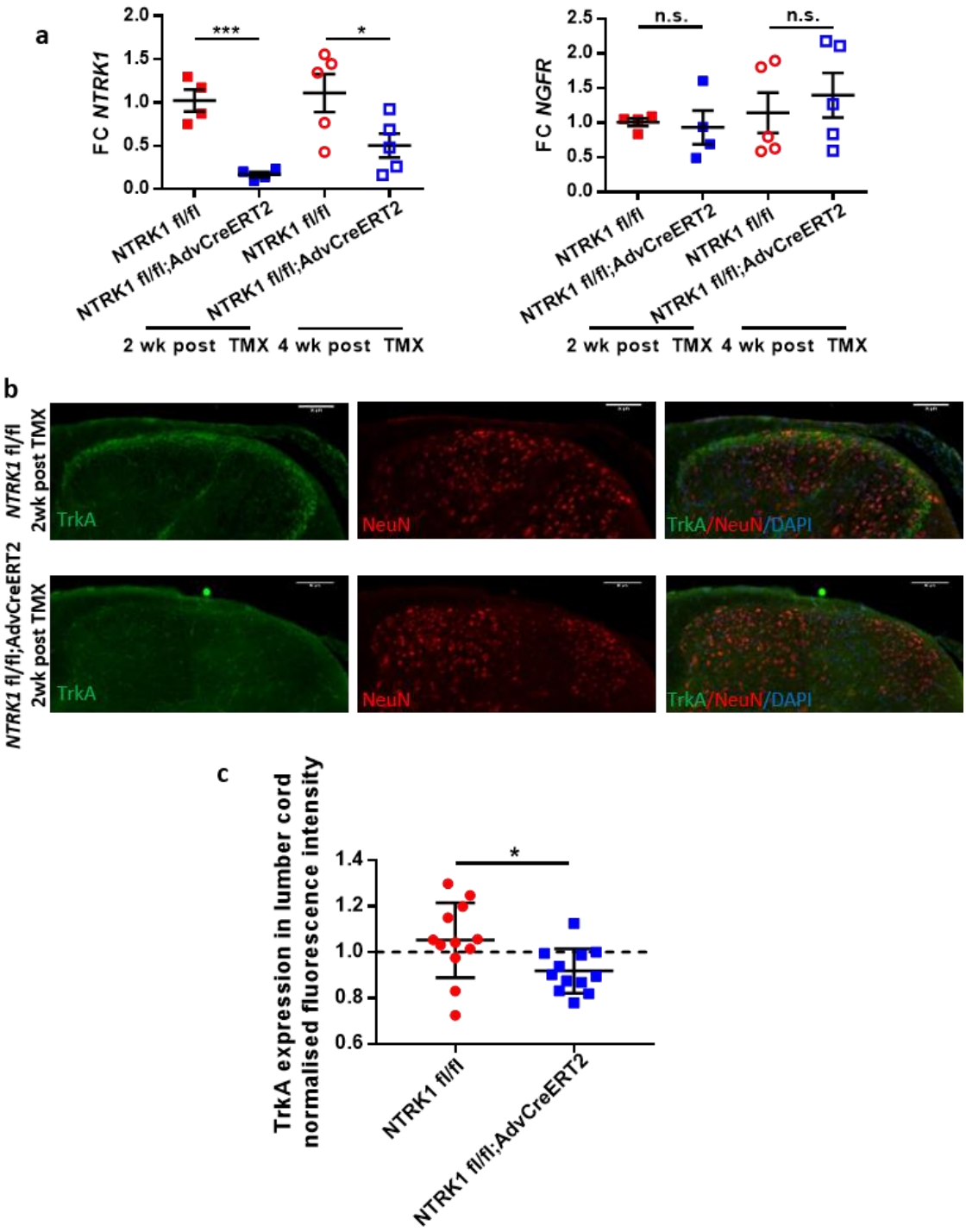


Figure 4.4 Tamoxifen injections in NTRK1 fl/fl; AdvCreERT2 mice results in reduced NTRK1 RNA levels in the DRG and TrkA protein expression in the lumbar spinal cord. a- *Fold change (FC) of NTRK1 and NGFR expression normalised to GAPDH levels and NTRK1 fl/fl control in NTRK1 fl/fl; AdvCreERT2 animals 2 (p=0.0006) and 4 (p=0.0456) weeks post i.p. TMX. Two*

tailed unpaired student t-test, n=4-5. ***b-*** *Representative immunohistochemistry of lumbar spinal cord dorsal horn from NTRK1 fl/fl and NTRK1 fl/fl; AdvCreERT2 mice 2 weeks post i.p. TMX. Stained with TrkA and pan-neuronal marker NeuN and* ***c-*** *quantification of TrkA expression (p=0.0228). Two tailed unpaired student t-test, n=4 animals (average from left and right dorsal horn from 3 sections, each normalised to background).*

4.3.3 There are no changes in peripheral skin innervation or cell size distribution of DRG neurons following knock out of TrkA *in vivo*

From previous studies investigating the effects of a global TrkA KO, changes have been observed in the periphery including differences in plantar skin innervation and a loss of small diameter neurons in the DRGs (Crowley et al. 1994; Smeyne et al. 1994). Therefore, following TMX mediated KO of TrkA, we investigated if there were any gross anatomical differences in skin innervation. We therefore measured the intraepidermal nerve fibre (IENF) density in the plantar skin of the hind paw by counting the number of sensory fibres crossing in the intraepidermal space as visualised by PGP 9.5 staining (Lauria et al. 2005). We observed no significant difference between the number of IENF normalised to *NTRK1* fl/fl littermates either 2 or 4 week post-TMX in *NTRK1* fl/fl; AdvCreERT2 mice, one-way ANOVA with Tukey post-hoc test. There was a fold change in the number of IENF from 1.00 ± 0.12 in *NTRK1* fl/fl compared to 1.10 ± 0.12 in *NTRK1* fl/fl; AdvCreERT2 2 weeks post TMX and a fold change from 1.00 ± 0.23 in *NTRK1* fl/fl to 1.29 ± 0.62 in *NTRK1* fl/fl; AdvCreERT2 animals 4 weeks post TMX, one-way ANOVA with Tukey multiple comparison (**Fig 4.5a and b**). Next, we measured the cell size distribution of neurons in the L4 DRG by measuring the cell diameter of neurons in sections. There was no significant difference between the *NTRK1* fl/fl; AdvCreERT2 and *NTRK1* fl/fl 2 and 4 weeks post TMX, two-way ANOVA with Sidak's post-hoc test (**Fig 4.5c and d**).

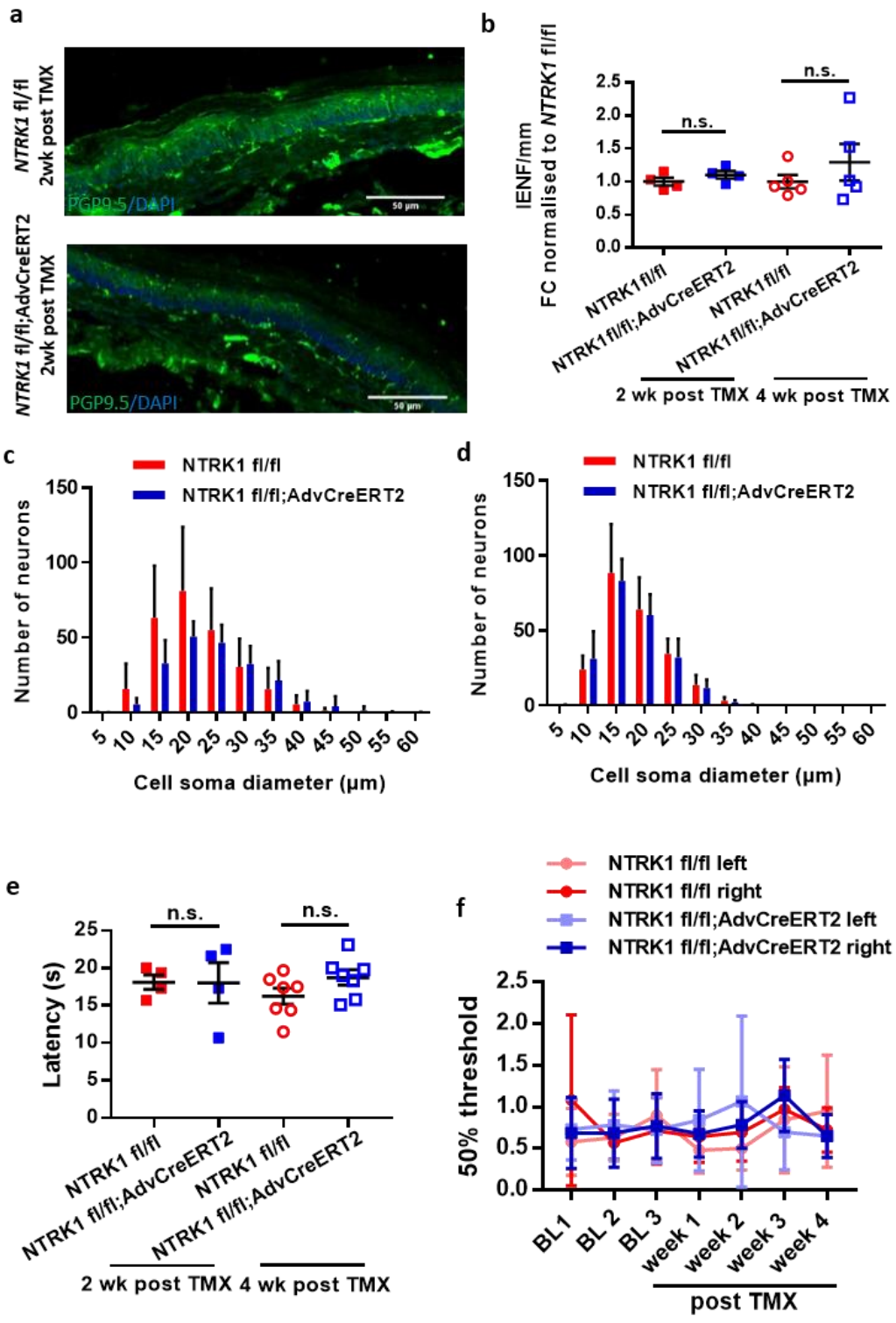


Figure 4.5 KO of TrkA does not result in any changes in IENF, cell size distribution in the L4 DRG or have any behavioural effect in the naïve animal. a- *Representative immunohistochemistry of hind paw plantar skin of NTRK1 fl/fl and NTRK1 fl/fl; AdvCre ERT2*

mice 2 weeks post IP TMX. Stained with pan-neuronal marker PGP 9.5. ***b-*** *Quantification of IENF/mm in NTRK1 fl/fl; AdvCreERT2 animals 2 and 4 weeks post TMX, represented as a fold change normalised to NTRK1 fl/fl controls. One-way ANOVA with Tukey post-hoc, n=4-5. Cell size distribution of L4 DRG in NTRK1 fl/fl and NTRK1 fl/fl; AdvCreERT2 animals* ***c*** *2 and* ***d*** *4 weeks post TMX. No significant difference observed, two-way ANOVA with Sidak's multiple comparison, n=4-5.* ***e-*** *static hot plate and* ***f*** *von Frey behavioural assessments of thermal and mechanical hypersensitivity respectively in NTRK1 fl/fl; AdvCreERT2 animals 2 and 4 weeks post TMX, n=4-7. Static hot plate all tests not significant, two tailed unpaired student t-test. Von Frey test showed all tests not significant, two way ANOVA with Tukey post-hoc test.*

4.3.4 Knock out of TrkA *in vivo* has no effect on thermal or mechanical thresholds in the naïve mouse

Previous studies using global TrkA KO mice showed a hyposensitivity to thermal and mechanical stimuli (Crowley et al. 1994). Therefore, we tested *NTRK1* fl/fl; AdvCreERT2 mice 2 and 4 weeks post TMX for thermal and mechanical hyposensitivity in the naïve state. In this study the naïve state refers to the absence of any inflammatory or neuropathic pain model being used following injection with TMX in order to investigate the effect of knockout of the *NTRK1* gene alone. We used the static hot plate to test thermal hyposensitivity by placing animals onto a plate pre-warmed to 52^{o}C and measuring the time taken for the animal to respond (latency to withdraw) by flicking, licking or biting of the hind-paw. We found no significant difference in the latency to withdraw between *NTRK1* fl/fl; AdvCreERT2 or *NTRK1* fl/fl littermates either 2 or 4 weeks post TMX, two-tailed unpaired students t-test. At 2 weeks post TMX *NTRK1* fl/fl showed a latency of 18.1 ± 2.0 seconds compared to 18.0 ± 5.4 seconds in *NTRK1* fl/fl; AdvCreERT2 mice. 4 weeks post TMX *NTRK1* fl/fl littermates showed a latency of

16.2 ± 2.8 seconds compared to 18.7 ± 2.7 seconds in *NTRK1* fl/fl; AdvCreERT2 mice (**Fig 4.5e**). We also carried out Von Frey behavioural test to assess mechanical hyposensitivity where animal threshold to mechanical stimuli was tested using different weight filaments applied to the hind-paw using the SUDO Von Frey up down method (Bonin, Bories, and De Koninck 2014). We observed no significant difference in the 50% withdrawal threshold up to 4 weeks post TMX compared to baseline. There was also no difference between left and right hind paws in either the *NTRK1* fl/fl or *NTRK1* fl/fl; AdvCreERT2 mice, two-way ANOVA with Tukey post-hoc (**Fig 4.5f**).

4.3.5 RNA sequencing reveals a number of differentially regulated genes following KO of TrkA *in vivo* in the naïve animal

Since there are no functional differences or changes in the plantar skin innervation between *NTRK1* fl/fl; AdvCreERT2 and *NTRK1* fl/fl littermates 2 or 4 weeks post TMX we further investigated the phenotype of our KO animals by investigating their transcriptional profiles. of the animals. Both L2, 3 and 5 lumbar DRGs of were collected from age matched 16 week old mice (6 male and 2 female) in *NTRK1* fl/fl and *NTRK1* fl/fl; AdvCreERT2 mice 4 weeks post TMX.

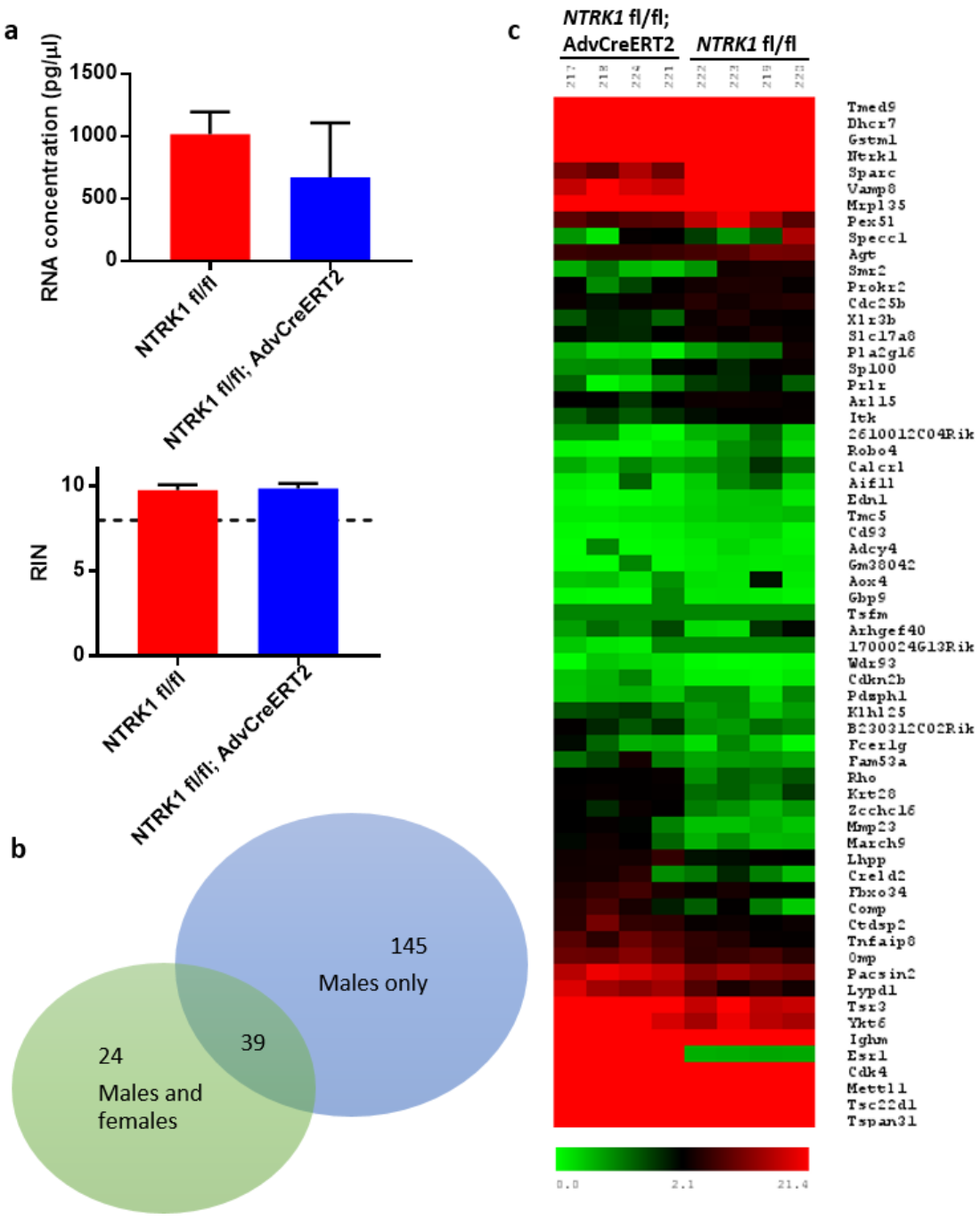


Figure 4.6 – RNA sequencing of DRGs isolated and MACS purified from NTRK1 fl/fl and NTRK1 fl/fl; AdvCreERT2 animals 4 weeks post TMX shows differential transcriptional profiles in naïve animals. a- *RNA concentration and RIN of the sequenced samples.* ***b-*** *Venn diagram showing the number of genes expressed in the analysis of combined samples from males and*

females or males alone. ***c-*** *Heat map showing expression of the genes identified from combined analysis of males and females.*

DRGs were triturated and MACS purified before RNA was isolated and purified. Firstly, the RNA was screened for quantity and quality (**Fig 4.6a**) before it was sent for RNA sequencing to Oxford genomics centre, University of Oxford. We received the data from the high throughput facility in the Oxford genomics centre and then realigned the data to the mouse genome and normalised to calculate the FPKM (fragments per kilobase of transcript per million fragments mapped) for each gene sequenced. Data was analysed using online bioinformatics tool the Galaxy project with the Tuxedo suit, where the sequence was aligned using the TopHat algorithm, the FPKM calculated using Cufflinks and then finally differences in the expression between samples from *NTRK1* fl/fl and *NTRK1* fl/fl; AdvCreERT2 animals using CuffDiff. Analysis of all collected samples revealed a total of 63 genes, 33 upregulated and 30 downregulated, significantly regulated between *NTRK1* fl/fl and *NTRK1* fl/fl; AdvCreERT2 mice (**Fig 4.6b and c**). Considering there might be a gender effect between the samples, we next excluded the two female samples. Analysis of the male samples by themselves revealed 184 genes (160 upregulated and 24 downregulated), regulated between *NTRK1* fl/fl; AdvCreERT2 and *NTRK1* fl/fl animals (**Fig 4.6b and Fig 4.7**). Comparing the mixed and the male only analysis showed only 39 differentially regulated genes common to both males and females and males alone, where 24 genes were specific to the males and females and 145 genes were specific to the males only.

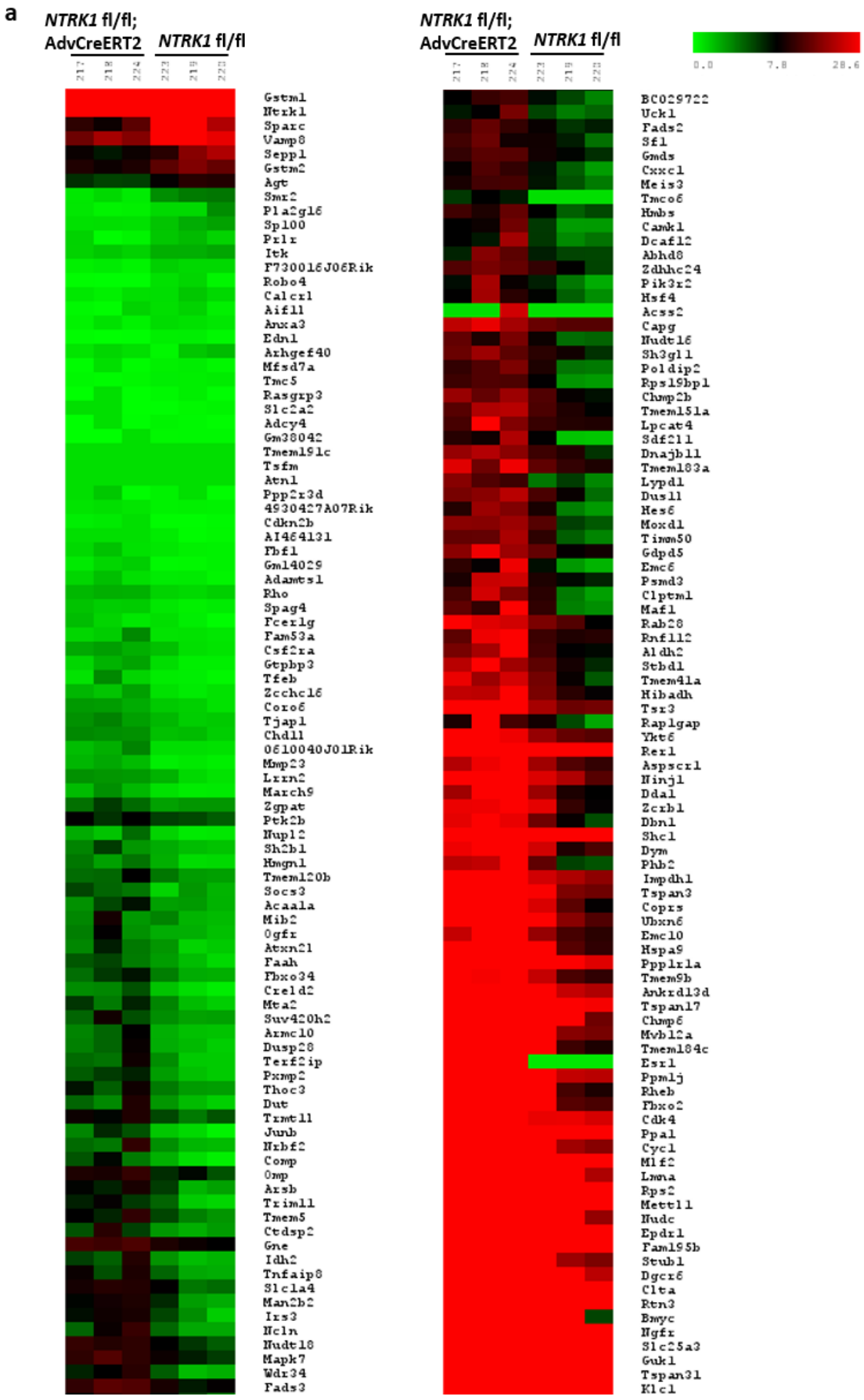

a
NTRK1 fl/fl; AdvCreERT2
NTRK1 fl/fl
217 218 224 223 219 220
Gstm1 Ntrk1 Sparc Vamp8 Sepp1 Gstm2 Agt Smr2 Pla2g16 Sp100 Prlr Itk F730016J06Rik Robo4 Calcrl Aifl1 Anxa3 Edn1 Arhgef40 Mfsd7a Tmc5 Rasgrp3 Slc2a2 Adcy4 Gm38042 Tmem191c Tsfm Atn1 Ppp2r3d 4930427A07Rik Cdkn2b AI464131 Fbf1 Gml4029 Adamts1 Rho Spag4 Fcer1g Fam53a Csf2ra Gtpbp3 Tfeb Zcchc16 Coro6 Tjap1 Chdl1 0610040J01Rik Mmp23 Lrrn2 March9 Zgpat Ptk2b Nup12 Sh2b1 Hmgn1 Tmem120b Socs3 Acaala Mib2 Ogfr Atxn21 Faah Fbxo34 Creld2 Mta2 Suv420h2 Armc10 Dusp28 Terf2ip Pxmp2 Thoc3 Dut Trmt11 Junb Nrbf2 Comp Omp Arsb Trim11 Tmem5 Ctdsp2 Gne Idh2 Tnfaip8 Slc1a4 Man2b2 Irs3 Ncln Nudt18 Mapk7 Wdr34 Fads3
NTRK1 fl/fl; AdvCreERT2
NTRK1 fl/fl
0.0 7.8 28.6
BC029722 Uck1 Fads2 Sfl Gmds Cxxc1 Meis3 Tmco6 Hmbs Camk1 Dcaf12 Abhd8 Zdhhc24 Pik3r2 Hsf4 Acss2 Capg Nudt16 Sh3gl1 Poldip2 Rps19bp1 Chmp2b Tmem151a Lpcat4 Sdf2l1 Dnajb11 Tmem183a Lypd1 Dus11 Hes6 Moxd1 Timm50 Gdpd5 Emc6 Psmd3 C1ptm1 Maf1 Rab28 Rnf112 Aldh2 Stbd1 Tmem41a Hibadh Tsr3 Rap1gap Ykt6 Rer1 Aspscr1 Ninj1 Ddal Zcrb1 Dbn1 Shc1 Dym Phb2 Impdh1 Tspan3 Coprs Ubxn6 Emc10 Hspa9 Ppp1r1a Tmem9b Ankrd13d Tspan17 Chmp6 Mvb12a Tmem184c Esr1 Ppm1j Rheb Fbxo2 Cdk4 Ppa1 Cyc1 Mlf2 Lmna Rps2 Mettl1 Nudc Epdr1 Fam195b Stub1 Dgcr6 Clta Rtn3 Bmyc Ngfx Slc25a3 Guk1 Tspan31 Klc1

Figure 4.7 – RNA sequencing on NTRK1 fl/fl and NTRK1 fl/fl; AdvCreERT2 animals 4 weeks post TMX shows differential transcriptional profiles in naïve male animals. a- *Heat map from RNA sequencing showing expression of the genes identified in analysis of male animals.*

4.3.6 Conditional KO of p75 *in vivo* results in a decrease in p75 protein and RNA transcript levels

We next characterised the effect of p75 knockout in *NGFR* fl/fl; AdvCreERT2 animals. Adult *NGFR* fl/fl; AdvCreERT2 and *NGFR* fl/fl littermates were i.p. injected with TMX for 3 days and L3 and L4 DRGs were collected 2 weeks after the first injection for RNA and immunohistochemical analysis respectively. A significant decrease in the expression of p75 in the L4 DRG was observed in *NGFR* fl/fl; AdvCreERT2 animals compared to *NGFR* fl/fl 2 weeks post TMX as assessed by immunohistochemistry staining of L4 DRG sections for p75 expression in neurons, $p<0.0001$ two-tailed unpaired student t-test. We quantified the p75 staining and found the percentage p75 positive neurons in *NGFR* fl/fl mice in L4 DRG was 53.1% ± 6.5% compared to 1.7% ± 0.5% in *NGFR* fl/fl; AdvCreERT2 mice 2 weeks post TMX (**Fig 4.8a and b**). Similarly, there was a significant decrease in the expression of *NGFR* gene normalised to *GAPDH*. There was a decrease from 1.02 ± 0.22 in *NGFR* fl/fl to 0.49 ± 0.05 fold change in *NGFR* fl/fl; AdvCreERT2 mice 2 weeks post TMX, $p=0.0101$ two-tailed unpaired student t-test. We then investigated if the KO of *NGFR* gene affected the expression of the *NTRK1* gene. There was no significant difference in the expression of *NTRK1* normalised to *GAPDH* where the fold change in *NGFR* fl/fl animals was 1.04 ± 0.34 compared to 0.92 ± 0.05 in *NGFR* fl/fl; AdvCreERT2 2 weeks post TMX (**Fig 4.8c**).

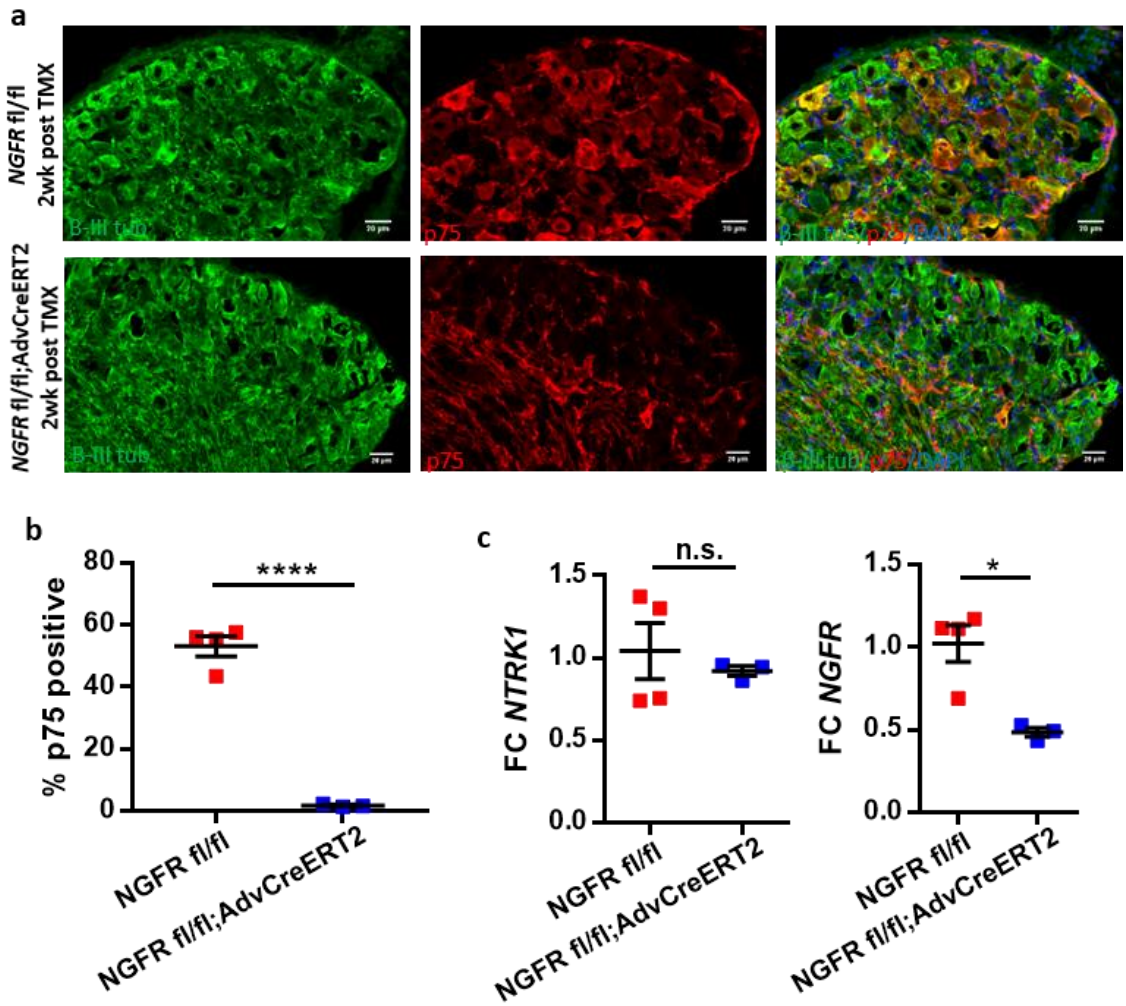


Figure 4.8 – Two weeks post tamoxifen injection there is reduction of p75 at the RNA and protein level in NGFR fl/fl; AdvCreERT2 animals. a- *Representative immunohistochemistry of a L4 DRG from NGFR fl/fl and NGFR fl/fl AdvCreERT2 mice 2 weeks post TMX. Stained for p75 and pan-neuronal marker β-III tubulin.* ***b-*** *Quantification of p75 expression 2 weeks post TMX ($p<0.0001$), two tailed unpaired student t-test, n=3-4.* ***c-*** *Expression of NTRK1 and NGFR ($p=0.0101$) 2 weeks post TMX in NGFR fl/fl and NGFR fl/fl; AdvCreERT2 animals. Levels of NTRK1 and NGFR expression were normalised to GAPDH expression before normalisation to NGFR fl/fl control and expressed as FC. Two-tailed unpaired student t-test, n=3-4.*

4.3.7 There are no changes in peripheral skin innervation or cell size distribution of DRG neurons following knock out of p75 *in vivo*

Comparable to TrkA global knockout mice, p75 global knockout mice exhibit loss in peripheral innervation compared to controls (Bergmann et al. 1997; K. F. Lee et al. 1992). Therefore, we investigated the effect of p75 knockout in *advillin* positive neurons on IENF density in plantar skin of the hind paw by measuring the number of IENF positively stained for PGP 9.5 crossing into

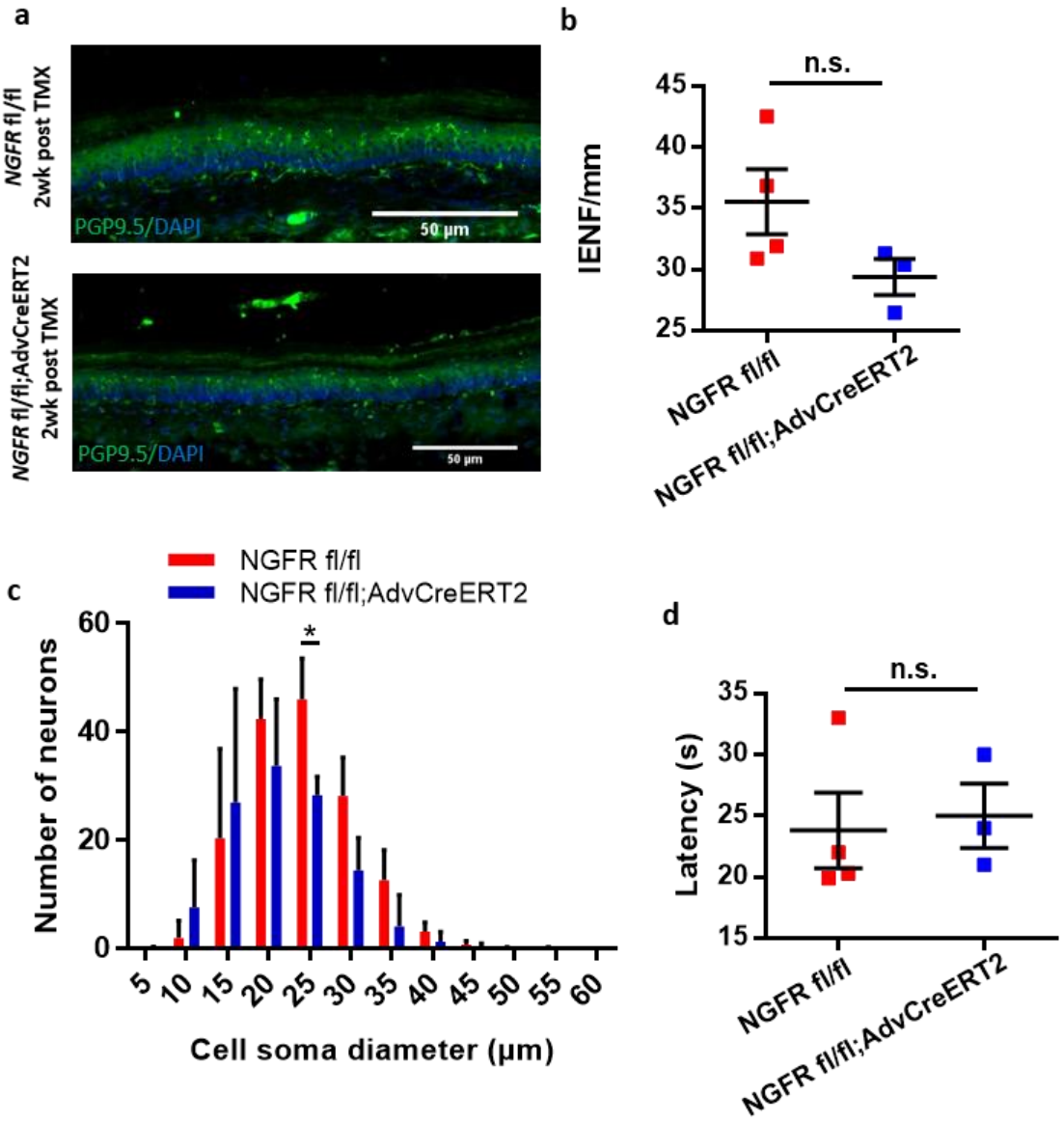


Figure 4.9 KO of p75 does not result in changes in IENF, cell size distribution in the L4 DRG or have any behavioural effect in the naïve animal. a- *Representative IHC of hind paw plantar skin of NGFR fl/fl and NGFR fl/fl; AdvCre ERT2 mice 2 weeks post i.p. TMX. Stained with pan-neuronal marker PGP 9.5.* ***b-*** *Quantification of IENF/mm in NGFR fl/fl; AdvCreERT2 animals 2 weeks post TMX. Two-tailed unpaired student t-test, n=3-4.* ***c-*** *Cell size distribution in L4 DRG in NGFR fl/fl and NGFR fl/fl; AdvCreERT2 animals 2 weeks post TMX (p=0.0181). Two-way ANOVA with Sidak's post-hoc, n=3-4.* ***d-*** *static hot plate assessment of thermal hypersensitivity in NGFR fl/fl; AdvCreERT2 animals 2 weeks post TMX. Static hot plate not significant, two tailed unpaired student t-test, n=3-4.*

the intraepidermal space. We observed no significant difference between *NGFR* fl/fl (35.5 ± 5.3 IENF/mm) littermates and *NGFR* fl/fl; AdvCreERT2 (29.4 ± 2.6 IENF/mm) mice 2 weeks post TMX, two-tailed unpaired student t-test (**Fig 4.9a and b**). As global KO for p75 have shown differences in the cell size distribution in DRG we then measured the cell size distribution in the L4 DRG and found no significant difference between *NGFR* fl/fl; AdvCreERT2 mice and *NGFR* fl/fl, with the exception of a significant decrease in the number of neurons with a cell soma diameter of 25µm, p=0.0181 two-way ANOVA with Sidak's post-hoc test (**Fig 4.9c**).

4.3.8 Knockout of TrkA *in vivo* has no effect on thermal threshold in the naïve mouse

Global p75 knockout animals have also been previously shown to be thermal hyposensitive (Bergmann et al. 1997; K. F. Lee et al. 1992). Therefore, we tested the hind paw of *NGFR* fl/fl; AdvCreERT2 mice on the static hot plate, where animals were placed onto a hot plate set to 52^{o}C and the time taken to respond by biting, licking or flicking of the hind paw was measured.

However, there was no significant difference between the latency to respond of *NGFR* fl/fl (23.8 ± 6.2 seconds) and *NGFR* fl/fl; AdvCreERT2 (25.0 ± 4.6 seconds) mice 2 weeks post TMX in naïve mice, two-tailed unpaired student t-test (**Fig 4.9d**). In this study the naïve mouse is described as an animal which has not been used in any neuropathic or inflammatory models following TMX mediated *NGFR* knockout.

4.4 Discussion

4.4.1 We generated new transgenic mouse lines to investigate the effect of knockout of the NGF receptors TrkA and p75

In the past several studies have investigated the effect of the global knockout of components of the NGF signalling pathway, including knockout of TrkA, p75 and NGF. In each of these knockout models animals exhibited insensitivity to noxious stimuli which was attributed to improper development of peripheral nerve fibres, as NGF is essential for the survival of sensory neurons until post-natal day 14 (Bennett et al. 1996; Lallemend and Ernfors 2012). In this study we wanted to avoid the effects of this early knockout of the NGF receptors. We therefore bred two new tamoxifen inducible transgenic mouse lines for the TrkA and p75 receptor. However, it turned out that these mouse lines had lower survival rates than anticipated. A significant number of animals that were positive for the Cre and the floxed gene were found dead for unknown reasons. Further investigation showed that there was no correlation between gender or age in animals found dead. This suggests that observed spontaneous death was not linked to the increased oestrogen levels in females or other sex linked differences nor linked to any specific developmental stage based on the age of animals. We observed two different peak times in the year that for increased spontaneous deaths. The peak from November 2015 until February 2016 correlated with the move of the colonies from one animal unit to another in November and then staff changes in the holding room. The peak

from March to May 2016 correlated with building works that took place to repair a service lift in the animal unit close to the holding room. Given that a significant percentage of the dead animals were positive for Cre and the floxed allele across the different AdvCre lines this could indicate that the double positive animals were more sensitive to stress or there could be "leakiness" of the Cre expression. However, global knockout lines for TrkA and p75 did not survive past weaning in the case of TrkA knockouts, or past 6 months in p75 knockout animals and exhibit an noticeable phenotype in relation to size and appearance of knockout animals compared to littermate controls (K. F. Lee et al. 1992; Bergmann et al. 1997; Smeyne et al. 1994). Therefore, it is unlikely that knockout of either receptor in the adult following activation of a "leaky" Cre would abruptly lead to death in these animals as mice appear physically normal. In the original study which generated and characterised the AdvCreERT2 mice less than 1% of cells were seen to have spontaneous Cre activation in control mice (Lau et al. 2011). One method to restrict the leakiness is to control the breeding of animals where you avoid the use of brother sister breeding pairs and limit the inheritance of the Cre recombinase to Cre positive males not females (Egan et al. 2007; Jackson Laboratory 2007; Heffner et al. 2012). In this study, we adhered to these rules where possible and used no brother sister breeding pairs, however, due to a limited number of available Cre positive animals from issues described in inheritance of the Cre and unexplained deaths some breeding pairs used Cre positive female mice. In order to test if any leakiness of Cre occurred in our animals we crossed the AdvCreERT2 mouse line with the TdTomato flox-STOP-flox reporter mouse with the aim to observe the expression of the TdTomato marker protein in both control and tamoxifen injected animals. This mouse line would reveal if there was TdTomato expression in non-tamoxifen injected animals and therefore indicate that our animals were indeed leaky. It would also shed light on which tissues express the TdTomato reporter protein in the tamoxifen injected animals. We expect expression to be limited to sensory and sympathetic neurons, but

we have not yet clarified this in our hands because breeding of these mice proved slow and due to time constraints it was not possible to breed a cohort of animals to perform experiments within this study. On the other hand this Cre mouse line has been previously used in the lab in combination with a floxed line for HDAC4, which as with TrkA and NGF, global knockout also results in underdeveloped animals which do not survive past weaning. However, there were no reports of unexplained deaths in these animals following crossing with AdvCreERT2 (Crow et al. 2015) suggesting that this Cre line has not exhibited these issues previously. However, in this earlier another batch of AdvCreERT2 mice from the same supplier was used generate the founder colony which unfortunately was not available in this study to cross with *NTRK1* fl/fl and *NGFR* fl/fl animals. Therefore, we cannot exclude the fact that the first batch of animals was slightly different to the second batch used here and that this difference might be causing the experienced higher mortality. Another explanation could be linked to the method of inheritance of the AdvCreERT2 gene. As described previously, the AdvCreERT2 mouse line was generated using a BAC which inserts at random sites in the genome (Lau et al. 2011). It is therefore possible in some animals it inserted the AdvCreERT2 gene into a critical part of the genome disrupting essential genes, however, if this was the case we would expect to see a higher number of unexplained deaths as all inheriting Cre in a specific site would be affected, unless a particular combination of Cre insertion sites into essential genes is required. In order to test this hypothesis of random insertion sites, whole genome sequencing would be necessary however this is beyond the scope of this thesis. Additionally, there are some reports from BSU staff of animals that were found dead showing evidence of seizures prior to death which could also be the causative factor for a number of unexplained deaths in this study.

4.4.2 Knockout of TrkA and p75 using the generated conditional knockout lines was successful at the RNA and protein level

We have first shown the successful knockout of TrkA in *NTRK1* fl/fl; AdvCreERT2 and p75 in *NGFR* fl/fl; AdvCreERT2 animals i.p. injected with TMX at the RNA and the protein level. At 2 and 4 weeks after TMX injection into *NTRK1* fl/fl; AdvCreERT2 animals there was a significant reduction in the percentage of TrkA positive neurons in the L4 DRG and superficial dorsal horn of the lumbar spinal cord 2 weeks following TMX injection, as assessed by immunohistochemistry. Taken together this suggests that 2 weeks after TMX maximal knockout of the *NTRK1* gene occurred and existing TrkA in the cell membrane has been turned over and intracellular stores depleted as assessed on the protein level, where TrkA turnover is dependent on activation by NGF followed by internalisation of the receptor and ubiquitin mediated degradation of TrkA (Geetha, Jiang, and Wooten 2005; Makkerh et al. 2005; Arévalo et al. 2006). We invested a fair amount of time and resources into the optimisation of the TrkA antibody staining, however, we were not successful until we used the antibody sold by R&D, which has been used for the staining presented in this study. As mentioned previously (chapter 4.3.2) the loxP sites of the *NTRK1* gene is located downstream of exons 1-3, which encode part of the extracellular region of the TrkA protein. It is therefore possible that knockout animals expressed a truncated TrkA protein. If this was the case this truncated version, although unlikely to be able to bind the ligand NGF and act as a scavenger receptor, it may still bind TrkA antibody and confound the results of TrkA staining. All of the TrkA antibodies tested in this study contain epitopes against a part of either the extracellular or the intracellular domain of the TrkA receptor. The TrkA antibody used in this study recognises an epitope at the start of the extracellular domain from amino acids 33 to 418 which overlaps with the TrkA region of any potential truncated protein from exon 1-3. We conclude that L4 DRGs from TMX injected *NTRK1* fl/fl; AdvCreERT2 animals show an ablation in the percentage of neurons positive for

TrkA staining and there is no evidence for the expression of a truncated form of TrkA (**Fig 4.3**). There is also evidence of a significant reduction in TrkA expression in the superficial dorsal horn of the lumbar spinal cord from TMX injected *NTRK1* fl/fl; AdvCreERT2 animals showing there is loss of TrkA protein from the terminals in addition to the soma (**Fig 4.4**). Similarly, to the *NTRK1* fl/fl; AdvCreERT2 animals, in *NGFR* fl/fl; AdvCreERT2 animals 2 weeks after TMX injection we observed an ablation in the percentage of neurons expressing the p75 receptor as assessed by immunohistochemistry. Contrary to the TrkA knockout mouse line the critical region of the *NGFR* gene in the p75 is exon 2 and there is no truncated protein predicted following excision of exon 2. Due to insufficient animals and time limitations we only measured knockout of p75 after 2 weeks after TMX injection.

RT-PCR performed on the RNA of whole DRG from both the *NTRK1* fl/fl; AdvCreERT2 and *NGFR* fl/fl; AdvCreERT2 animals confirmed that there was a reduction in the *NTRK1* gene expression and *NGFR* gene expression respectively following TMX injection, whereas the expression in the non-targeted receptor did not change. Therefore, we exclude the presence of a compensatory mechanism between the two NGF receptors in both mouse lines tested. Even though at the protein level we found an ablation of the protein of interest in both mouse lines, the RNA expression shows only a partial, though significant, decrease. However, this does not reflect a true partial decrease in the gene of interest in the sensory neurons, but rather stems from the fact that we isolated RNA from whole DRG which also include numerous glial cells (Richner et al. 2014), in addition to the gene of interest only expressed on a subset of sensory neurons. However, even though there was a partial decrease in both mouse lines it has been found that the *NGFR* gene but not the *NTRK1* gene is expressed in non-neuronal cells (chapter 1.1) (Gosselin et al. 2014; Lavin et al. 2014; Mo et al. 2015). Recombination of the loxP sites of the *NTRK1* gene results in a deletion of exons 4-6, therefore exon 1-3 are still transcribed and therefore might be detected. In this study we designed and tested a number of RT-PCR primers

targeted to different sections of the *NTRK1* transcript (chapter 3.2.11). In this study however, we used primers targeted to exons downstream of the floxed exons 4-6, therefore, as these primers do not bind exons 1-3 it is unlikely that the partial knockout of *NTRK1* gene expression observed here is the result of partial mRNA transcript. Another approach would be to MACS purify DRG from injected animals and isolate the RNA from these neurons enriched cultures and measure expression of the *NTRK1* and *NGFR* genes (chapter 3.2.9). Alternatively, *in situ* hybridisation could be used as a more sensitive technique to stain sections from DRGs from TMX injected *NTRK1* fl/fl; AdvCreERT2 or *NGFR* fl/fl; AdvCreERT2 animals to investigate RNA transcript levels of the *NTRK1* and *NGFR* genes respectively in neurons. However, the decrease in the percentage of neurons positive for the TrkA or p75 receptor protein suggests that there is effective knockout of the gene of interest, only limited by the detection limit of the antibodies that were used to detect TrkA and p75.

4.4.3 We found no changes in peripheral skin innervation or cell size distribution of neurons in *NTRK1* and *NGFR* KO animals

Studies investigating changes in global KO of NGF or the NGF receptors TrkA or p75 showed KO of either the ligand or the receptor to result in a decrease innervation of peripheral skin and a decrease in the total number of neurons and overall size of DRG, with a shift towards medium and large diameter neurons following a loss in small diameter neurons (Crowley et al. 1994; Smeyne et al. 1994; K. F. Lee et al. 1992; Bergmann et al. 1997). It is known that NGF is required for survival of sensory and sympathetic neurons during development and therefore the above mentioned anatomical changes in the animals are most likely the result of the knockout of NGF signalling during a critical point in development (Crowley et al. 1994; Smeyne et al. 1994; K. F. Lee et al. 1992; Bergmann et al. 1997). In order to bypass these developmental implications we bred conditional knockout animals. To investigate if there were

anatomical changes in adult *NTRK1* fl/fl; AdvCreERT2 or *NGFR* fl/fl; AdvCreERT2 animals injected with TMX we measured the innervation of plantar skin of the hindpaw and cell size distribution within the DRG. However, compared to controls there was no significant difference in plantar skin innervation in either *NTRK1* fl/fl; AdvCreERT2 or *NGFR* fl/fl; AdvCreERT2 knockout animals. We also observed no significant difference in the cell size distribution of DRG between control and knockout animals. Even though we did not measure the total number of neurons in the L4 DRGs there was no gross differences in the size of DRG during collection. Overall, from these preliminary experiments we concluded that knockout of either NGF receptor in the adult mouse did not result in any gross anatomical differences in the peripheral nervous system, supporting the current belief that while NGF is essential during development for the formation and survival of sensory neurons, this is not the case in the adult system (Patel et al. 2003).

4.4.4 There are no behavioural changes in thermal or mechanical sensitivity in either TrkA or p75 knockout animals

In addition to the decreased innervation of the plantar skin and loss of small diameter neurons in the DRG, global knockout mice for NGF, TrkA and p75 exhibit a hyposensitivity to thermal and mechanical stimuli as mentioned previously (chapter 1.2)(Crowley et al. 1994; K. F. Lee et al. 1992; Bergmann et al. 1997; Smeyne et al. 1994). It is believed that this hyposensitivity is the direct result of the loss of the sensory neurons in global KO animals following the loss of NGF signalling during development. In the adult however, there is increasing evidence for a role of NGF in pain (chapter 1.4), supported by a number of clinical trials using anti-NGF antibodies which have shown a significant reduction in pain, particularly in osteoarthritis (chapter 1.5) (Lane et al. 2010).

Since there in no role for NGF signalling in the adult mouse reported except following injury or inflammation, we hypothesised that in the adult mouse knockout of either the TrkA or the p75 alone would not exhibit no differences in pain related behaviour. Therefore, to characterise this mouse we investigated how the loss of either the TrkA or p75 receptor alone affected mouse behavioural response to mechanical and thermal stimuli in a pilot study using a small number of either *NTRK1* fl/fl; AdvCreERT2 or *NGFR* fl/fl; AdvCreERT2 against Cre negative littermate controls injected with TMX to see if there was any change in the "naïve" state. In this study, we refer to the naïve mouse as an animal which had not been tested using any inflammatory or neuropathic pain model. In an initial pilot with both *NTRK1* and *NGFR* KO animal lines a small cohort of knockout and control animals was used to measure thermal sensitivity using the static hot plate test 2 weeks after TMX injection. We found that in both *NTRK1* fl/fl; AdvCreERT2 and *NGFR* fl/fl; AdvCreERT2 animals there was no significant difference in the latency to respond between knockout and control animals. We did not perform any other behavioural tests with the *NGFR* fl/fl; AdvCreERT2 line due to limitations in the number of animals available for experiments. Since we were able to obtain a second, larger cohort of *NTRK1* fl/fl; AdvCreERT2 animals. We tested them for mechanical sensitivity using Von Frey hairs for 4 weeks after TMX injection, at which point they were also tested again for thermal sensitivity using the static hot plate. The aim of testing this second cohort was to investigate the effect of a longer period of TrkA knockout, allowing all the pre-formed protein to be turned over and the complete knockout to become effective. Again, we observed no evidence for thermal or mechanical hypersensitivity of knockout animals compared to controls. The initial plan for this study was to test animals for up to 6 weeks after TMX injection, however due to several unexplained deaths in this cohort of animals and the risk of not having enough animals survive the end of the study we chose to shorten the post TMX injection to four weeks. Due to the unexpected deaths in this breeding line that we discussed

before, we assumed that the dying animals post TMX injection were also Cre positive floxed animals. This was later confirmed, through genotyping of earclips taken after the study, to be true. Except for these behavioural tests we did not perform any other tests, however we observed no obvious phenotype in these animals. In this study we carried tests for the most likely behavioural deficits suggested from studies using global knockout lines. However, to fully characterise these animals a broader range of tests was required, for example cold plate for cold thermal sensitivity (Brenner et al. 2015; Brenner, Golden, and Gereau IV 2012), open field test to investigate the anxiety general locomotion (Carola et al. 2002) and rotarod to test motor impairment (Shiotsuki et al. 2010). Unfortunately, this was outside of the scope of this thesis. A longer study characterising the effect of p75 knockout in adult animals would also be interesting to perform, however, as previously mentioned it was not possible to obtain the necessary number of animals within the scope of this thesis. In general, working with conditional knockout mouse lines has the disadvantage that larger numbers of animals are needed to obtain the correct genotype for testing behavioural outcomes of the knockout. In our case, this was even more aggravated by the fact that Cre positive floxed animals had a higher mortality rate. Furthermore, we observed that the inheritance of the Cre gene following crossing of the AdvCreERT2 and floxed animals did not follow Mendelian law. Overall only 30% of the offspring were Cre positive, resulting in higher numbers of breeding pairs needed in studies for behavioural testing. These circumstances forced us to pool animals from a wider age range than normally aimed for, from 9-19 weeks across cohorts. Where possible we age-matched animals between control and knockout animal groups. We believe that these rather large age ranges within the experiment may have a confounding effect on the result from this study. Animals from 12-24 weeks are considered adult animals (NIF 2010). Since the youngest animals of our study were 9 weeks old when TMX was injected and therefore still adolescent and the oldest were 23 weeks at the end of this study when they were behavioural assessed, it

is reasonable to believe that age may have been a confounding element in the study. In addition, our behavioural cohorts were mixed genders, which could have been a further confounding element to this study. To avoid this larger animal colonies would have to be bred. Overall, we found in this study with the animal numbers that were available to us that KO of either the TrkA or p75 alone did not result in any mechanical or thermal hypersensitivity at the time point post TMX injection that we tested.

As mentioned above there is evidence for a role of NGF in pain in the adult (chapter 1.4). Phase II and III clinical trials involving treatment with anti-NGF antibodies have shown a decrease in pain in a number of different pain conditions including osteoarthritis, lower back pain and diabetic polyneuropathy and bladder pain syndrome (Lane et al. 2010; Bannwarth and Kostine 2014; Capsoni and Cattaneo 2006; Aloe et al. 2012). To test for a pain phenotype in our *NTRK1* fl/fl; AdvCreERT2 or *NGFR* fl/fl; AdvCreERT2 mouse lines, animals could be challenged in inflammatory and neuropathic pain models. Previously in preclinical models, studies using where animals were exposed to inflammatory models such as CFA injection into the hind-paw, injections of anti-NGF reversed the onset of thermal and mechanical hypersensitivity (Andreev et al. 1995; Lewin, Ritter, and Mendell 1993; McMahon et al. 1995; Woolf et al. 1994). Therefore, we hypothesis that in animals with KO of either the TrkA or the p75 receptor prior to exposure to inflammatory or neuropathic pain models after TMX injection there will be a reduction in the pain phenotype for example in behavioural tests measuring thermal or mechanical sensitivity in knockout animals compared to controls. Animals could be tested using inflammatory pain models such as injection of complete Freud's adjuvant (CFA) into the hind-paw (Fehrenbacher, Vasko, and Duarte 2015), neuropathic pain models such as sciatic nerve ligation (SNL) (Yalcin et al. 2014; Malmberg and Basbaum 1998) or disease models such as the arthritis MIA model (Rahman and Dickenson 2015; Sun, Beier, and

Pest 2017). Together, these experiments will further shed light on the role of NGF signalling in neurons in the context of pain following neuron specific knockout of the NGF receptors.

4.4.5 RNA sequencing of *NTRK1* KO animals shows changes in gene expression

Next we performed RNA sequencing analysis on RNA isolated from MACS purified L2, 3 and 5 DRG of *NTRK1* fl/fl; AdvCreERT2 animals, 4 weeks after TMX injection. Our preliminary analysis showed that 63 genes were differentially regulated in samples where genders were mixed compared to 184 differentially expressed genes in males. This suggests an influence on the gender on the adaptation to the knockout of the TrkA receptor via TMX injection. For example, the females might have been more susceptible to tamoxifen compared to males which could be assessed by comparing differences in gene expression in males and females across the knockout (TMX injected Cre positive *NTRK1* floxed animals) and the control groups (TMX injected Cre negative *NTRK1* floxed animals) used in this experiment. However, the mixed group did not consist of equal males and females, but rather consisted of 6 males and 2 females, so this hypothesis of a gender difference could not be tested. For example, recently there is increasing evidence that there are gender specific mechanisms in pain sensation, where a study by Sorge *et al* observed that compared to males, microglia in female mice are not required for the development of mechanical hypersensitivity in chronic pain. Instead female mice exhibit an increase in the number of T cells suggesting that in female mice chronic pain is likely the result of a mechanism involving the adaptive immunity (Sorge et al. 2015). However, animals used in this study were age matched, 19 weeks at the time of injection, and taken from the cohort used for thermal and mechanical hypersensitivity described above where there was no difference observed in behaviour which could explain gender differences observed from the RNA sequencing. Gene comparison between the mixed and male only group has shown that only 39 genes were differentially expressed. These genes have a high potential to be connected to TrkA knockout. In addition to comparing the number of genes

differentially expressed we also performed protein-protein interaction analysis using the online software STRING. This software identifies protein networks which the differentially expressed genes could be part of. However, we found that there were no significant networks of proteins regulated in TrkA KO animals. Further analysis could include grouping genes into protein functionality could also be a useful tool to identify the range of regulated genes, for example the number of genes either upregulated or downregulated that code for transcription factors vs signalling molecules vs structural proteins. This data set harbours a high potential to identify novel gene that are part of the NGF signalling pathway. However, in-depth analysis of this dataset was beyond the scope of this thesis. In the future, potential novel genes also require validation by PCR and recurrence in a separate cohort of animals.

We first investigated *NTRK1* gene expression in samples from *NTRK1* fl/fl; AdvCreERT2 animals compared to controls. Reassuringly, we found *NTRK1* in both analyses (male and females mixed and males only) to be significantly decreased in knockout animals. However, in the mixed group the p value was $p<10^{-5}$ and in the male group it was $p<10^{-3}$. This suggests that in the male group there was residual *NTRK1* RNA. There are several different explanations for this. First, there could be residual mRNA left in the cells which has not been degraded. Second, as mentioned previously (chapter 3.1.1), the loxP sites are positioned downstream of exon 1-3, around exons 4-6, which might result in a truncated RNA fragment. We tested this by further analysing the alignment of the results from the RNA sequencing to the genome using the Galaxy software. However, we found that a significant proportion of the fragments for TrkA sequenced to be downstream of the floxed region, which makes this hypothesis unlikely. Furthermore, the TrkA RNA is similar in sequence to the other Trk receptors TrkB and TrkC. The realignment algorithm used post RNA sequencing deals with transcripts of areas common to several proteins, such as protein kinase domains, in the following ways. The total number of reads is divided between the number of genes containing this region. Therefore, it is possible

that some of the reads assigned to TrkA in these animals could in fact be from other similar proteins, such as TrkB or TrkC. Finally, we received the AdvCreERT2 mouse line as a generous gift from John Wood, University College London. It has been reported that due to the hemizygous nature of these mice the inheritance of the transgene is not stable (Jax 2017), which is in line with the findings of this study of only 30% inheritance (**Fig 4.2**). This fact suggests that each generation shall be screened for the number of copies of the AdvCreERT2 gene in each animal (Jax 2017). There might be a dose response in the efficiency of gene knockout correlating to the number of AdvCreERT2 copies. In the original publication characterising this Cre mouse line, copy number was assessed by comparing the intensity of bands on southern blots taken from wildtype and mutant mice (Lau et al. 2011), however copy number can also be assessed using qRT-PCR (Luo et al. 2014).

However, preliminary data from the analyses has shown a couple of interesting genes with different expression patterns. These include Cdk4, cyclin dependent kinase 4, which is involved in cell cycle regulation and is used as a target in cancer. Interestingly it has also been found to have a role in spinal cord injury (Yang et al. 2014; J Wu et al. 2014; Junfang Wu et al. 2013). March9, membrane-associated ring finger (C3HC4) 9, which is a membrane-bound E3 ubiquitin ligase which targets the internalisation of several membrane glycoproteins (Hör et al. 2009; Hoer, Smith, and Lehner 2007; Jahnke, Trowsdale, and Kelly 2013) and Vamp8, vesicle associate membrane protein 8, which encodes an integral membrane protein belonging to the synaptobrevin/ VAMP subfamily of soluble N-ethylmaleimde-sensitive factor attachment protein receptors (SNAREs) which is involved in the fusion of vesicles with the presynaptic membrane (Marshall et al. 2015; Behrendorff et al. 2011).

The big drawback of this analysis is that with the exception of CGRP ($p< 10^{-3}$) we did not observe any changes following *NTRK1* KO in the gene expression of known proteins downstream of NGF signalling, such as substance P or TRPV1 (Pezet and McMahon 2006;

Dubin and Patapoutian 2010; A. Patapoutian, Tate, and Woolf 2009). However, as these genes are commonly believed to be pain related genes it is not surprising that we do not observe changes in these genes in the naïve state.

4.5 Supplementary material

Antibody	Target in protein/ Antigen	Dilution	Use	Tissue	Fixation	Comments
Biosensis R-152-100	Extracellular domain human TrkA	1:250, 1:500 and 1:1000	ICC	WT mix and MACS DRG culture	2/4% PFA	Faint neurite staining, autofluorescence in the soma
		1:500	IHC	WT DRG	1, 2 and 4% fixation	4% more autoflourescence, no difference following biotin amplification
		1:500	IHC	WT DRG	Snap frozen and 2% perfused and non-perfused	High levels of background across DRG, slight gradient could represent "positive" cells as brighter
ab76291	Intracellular cytoplasmic domain, residues 445 and 495 of human TrkA	1:250	ICC	MACS purified cells	2% PFA	Staining matched secondary only control
		1:100 1:500	IHC	WT DRG	Snap frozen and 2% perfused and non-perfused	Snap frozen no staining, some with 2% fixation for both [Ab]
		1:500	IHC	TMX treated control and *NTRK1* CKO L4 DRG	2% PFA	Positive staining in the control and the CKO animals
ab109010	Synthetic peptide corresponding to residues in human TrkA	1:250, 1:500 and 1:1000	ICC	MACS purified cells	2% PFA	Very few "positive" stained cells even at higher concentration
		1:500		WT DRG	1, 2 and 4% PFA fixation	Increasing background with increasing concentration of PFA. Biotin amplification saturated signal.
		1:500	IHC	WT DRG	Snap frozen and 2% perfused and non-perfused	Few in the snap frozen, similar levels in the 2% perfused ones. Seems to be a gradient in the levels that are positive for TrkA
		1:500	IHC	TMX treated control and *NTRK1* CKO L4 DRG	2% PFA	Positive staining in the control and the CKO animals
ab36961	Extracellular domain of Human TrkA	1:1000	IHC	WT DRG	1, 2 and 4% fixation	Poor staining across the DRG, very faint, no increase in staining following biotin amplification.
Biosensis BS292	Intracellular cytoplasmic domain human TrkA, aa440-796	1:500 and 1:1000	IHC	WT DRG	2% PFA fixation	Very few cells and high background staining, staining of SGC surrounding neurons.
		1:500 and 1:1000	IHC	rat L DRG	2% and 4% PFA	No staining
Biosensis BS293	Extracellular domain of human TrkA	1:500 and 1:1000	IHC	WT DRG	2% PFA	Only stained SGC surrounding the neurons.
		1:500 and 1:1000	IHC	rat L DRG	2% and 4% PFA	No staining
Biosensis BS315	Intracellular domain of human TrkA	1:500 and 1:1000	IHC	WT DRG	2% PFA	Only stained SGC surrounding the neurons.
		1:500 and 1:1000	IHC	Rat L DRG	2% and 4% PFA	Only stained SGC surrounding the neurons.
Biosensis BS470	Extracellular domain human TrkA aa33-432	1:500 and 1:1000	IHC	WT DRG	2% PFA	Only stained SGC surrounding the neurons.
		1:500 and 1:1000	IHC	Rat L DRG	2% and 4% PFA	Some staining but faint and punctate
R&D af1056	Mouse myeloma cell line NS0-derived recombinant rat TrkA Ala33-Pro418	1:200	IHC	TMX treated control and *NTRK1* CKO L4 DRG	2% PFA	Positive staining in the control animals and none in the CKO animals

Supplementary table 4.1 Details of the different TrkA antibodies tested using immunocytochemistry (ICC) and immunohistochemistry (IHC).

Chapter 5: General discussion

5.1 Main conclusions of the study

NGF has been identified to have a role in the development of the sensory and sympathetic nervous system (K. F. Lee et al. 1992; Bergmann et al. 1997; Crowley et al. 1994; Smeyne et al. 1994). In adulthood on the other side there is evidence for a role for NGF in pain (Andreev et al. 1995; Lewin, Ritter, and Mendell 1993; Bennett et al. 1998; McMahon et al. 1995; Woolf et al. 1994). It has been shown that NGF is important for peripheral and central sensitisation in addition to hyperinnervation of tissues in the development of pain (Kelleher, Tewari, and McMahon 2016; Dubin and Patapoutian 2010; A. Patapoutian, Tate, and Woolf 2009; Kerr et al. 1999; Michael et al. 1997; Jimenez-Andrade et al. 2011). Preclinical rodent models and phase II and III clinical trials using anti-NGF therapies have shown success in the alleviation of pain (Lane et al. 2010; Bannwarth and Kostine 2014; Aloe et al. 2012; Capsoni and Cattaneo 2006), however, studies using global knockout mouse lines for either NGF or its receptors TrkA and p75 to investigate the role of NGF in pain have failed to address the precise role of NGF in pain. Global knockouts do exhibit hyposensitivity to thermal and mechanical stimuli, however these defects are attributed to the effects of loss of NGF during development rather than to its role in the adult animal (Crowley et al. 1994; Levi-montalcini 1987).

The aim of this study was to investigate unanswered questions concerning the mechanism of action of NGF in pain. First, we aimed to answer if NGF binds directly to neurons or exhibits its effects indirectly via non-neuronal cells, which have been shown to express receptors for NGF (Richner et al. 2014; Thakur et al. 2014; Usoskin et al. 2014; Aloe et al. 2012; Gosselin et al. 2014; Lavin et al. 2014; Mo et al. 2015; Ebenezer et al. 2007). For this we optimised and

characterised a method of purifying neuronal cultures using MACS purification of adult DRGs. Second, we set out to identify the main receptor for NGF in pain between TrkA and p75. We therefore obtained knockout mouse lines, *NTRK1* fl/fl and *NGFR* f/fl, in which critical regions in the *NTRK1* gene for TrkA and the *NGFR* gene for p75 are floxed and conditional knockout is induced through the action of Cre. We used both lenti and adeno-associated viruses to characterise their efficiency for Cre mediated excision of floxed genes and in parallel bred a novel inducible conditional knockout mouse line for these NGF receptors by crossing the floxed lines with an *Advillin*-CreERT2 mouse line to generate sensory neuron specific knockout animals under the control of tamoxifen.

We described in this study that MACS purification is an effective method in purifying viable DRG neurons to a greater level of purity and using a less labour intensive and faster protocol than previous methods used including immunopanning and FACS. However, we also identified a disadvantage of this method since there is a loss of medium and large diameter neurons. Large diameter neurons consist of low threshold, myelinated, mechanoreceptors sensitive to non-noxious stimuli with a role in proprioception and fine touch, whereas medium diameter neurons include medium threshold thinly myelinated fibres such as Aδ fibres (Hudspith, Siddall, and Munglani 2006; Richner et al. 2014). This project investigated the role of NGF in pain and therefore focuses on primary afferent nociceptors which include small diameter high threshold, unmyelinated C-fibres – specifically peptidergic nociceptors - and small-medium diameter myelinated Aδ fibres, which both express TrkA and p75 receptors (Hudspith, Siddall, and Munglani 2006; Richner et al. 2014). Therefore, for this study, the use of MACS purification is suitable. Through the characterisation and optimisation of this method we developed a protocol with which pure neurons can be isolated and used to test the direct effect of factors such as elements of the inflammatory soup secreted in pain, for example NGF, which can then either be compared to non-MACS purified cultures or used later to set up co-

cultures where an indirect effect via non-neuronal cells may be observed. We have achieved the first aim of the project to develop a method to study the direct effect of NGF on neurons.

The second question was to investigate whether NGF in pain signals mainly through the TrkA or the p75 receptor. To address this, we used transgenic mouse lines with LoxP flanked regions of either the *NTRK1* gene for TrkA or *NGFR* gene for p75. We employed two different viral approaches to knockout these genes and we generated a new mouse line for conditional tamoxifen dependent knockout of TrkA or p75 in sensory neurons. We determined in this study that the treatment of MACS purified cultures with lenti Cre-GFP virus was not optimal for knockout of either the TrkA or the p75 NGF receptors. We observed that after three days in culture, the optimal time determined for efficient transduction of cells and therefore knockout of the floxed gene of interest, a ceiling effect in neuronal response to NGF was observed, as measured using a neurite outgrowth assay. We found no difference in neurite outgrowth between no NGF controls and cultures treated with NGF suggesting that after 3 days the levels of NGF had reached saturation following secretion from neuronal cells, resulting in insensitivity of the neurite outgrowth assay to observe any differences in early NGF treatment of cultures. Therefore, we chose to use an alternative method of viral transduction using IT delivery of AAV9 Cre-eGFP to knockout TrkA and p75 in floxed mice *in vivo* prior to culturing MACS purified neurons from DRG. We found that AAV9 mediated knockout of both the TrkA and the p75 receptor was successful on the protein level however at the RNA level the knockout of the *NTRK1* gene was not as clear. There was a trend towards a reduction in the expression of the *NTRK1* gene in *NTRK1* fl/fl animals 2 or 4 weeks after AAV9 Cre-eGFP injection, and no change in the expression of the *NGFR* gene. This suggests that knockout of TrkA in these animals did not result in any compensatory upregulation in p75. It is important to note that only a small sample size was used to measure gene expression changes by RT-PCR. It would therefore be preferable to increase the sample size to add further statistical power to this result. In *NGFR*

fl/fl animals there was a significant reduction in the expression of both the *NTRK1* and *NGFR* genes 2 weeks after injection of AAV9 Cre-eGFP. This suggests that following the knockout of p75 there is a mechanism activated that leads to a decrease in *NTRK1* expression. However, in this study we also characterised the knockout of p75 receptor using a novel conditional mouse model by crossing *NGFR* fl/fl mice with an AdvCreERT2 mouse line, where knockout of the floxed gene in sensory neurons is controlled by tamoxifen. Two weeks after tamoxifen treatment of *NGFR* fl/fl; AdvCreERT2 mice we observed a significant decrease in the percentage of neurons expressing p75 neurons (as assessed by immunohistochemistry), corresponding with a significant decrease in the expression of the *NGFR* gene at the RNA level using RT-PCR. However, in this model we did not observe a decrease in *NTRK1* expression (as has been observed in neurons isolated from *NGFR* fl/fl mice injected with AAV9 Cre-eGFP). This conflicting result could be firstly the result of a decreased statistical power in the AAV mediated knockout compared to the transgenic mouse model. This could be tested by increasing the number of samples tested. Or secondarily due to the route of knockout. The adeno-associated viral particles could have a side effect. This could be investigated we think by including controls such as AAV9 eGFP without Cre expression in floxed animals or AAV9 Cre-eGFP in WT animals. Thirdly, treatment with tamoxifen could have also resulted in side effects. This could be addressed using tamoxifen injections in WT animals.

Expression of the *NTRK1* and *NGFR* genes and percentage of neurons positive for TrkA protein was also assessed in *NTRK1* fl/fl; AdvCreERT2 mice treated with tamoxifen. Compared to *NTRK1* fl/fl animals injected with AAV9 Cre-eGFP where the percentage of cells expressing TrkA was measured in L4 DRG sections, there was a comparable reduction in the percentage of neurons positive for TrkA protein following tamoxifen. There was also no change in the expression of the *NGFR* gene. However, contrary to the AAV9 model *in vivo* we found a significant decrease in *NTRK1* expression (compared to a trend towards a decrease with AAV9).

We concluded that knockout of floxed genes using either AAV9 mediated Cre delivery or *in vivo* Cre recombinase activated by tamoxifen results in successful in knockout on the protein level and the RNA level. However, there are confounds to both methods. Using the viral approach with intrathecal delivery exposes animals to a surgical procedure and there is the risk of spinal cord damage during this process. There is also the issue of high variability of transduction both between animals and within animals, as we observed that there is a degree of bias to laterality in transduction making this method unsuitable for behavioural studies. However, by using the GFP marker protein, this model is useful when using cells in cultures since each experiment has its own internal controls. On the other hand, the novel inducible condition knockout lines for the *NTRK1* and *NGFR* genes bred in this study have demonstrated to be difficult to breed due to the occurrence of abnormally high numbers of unexplained deaths. This results in costlier and more time-consuming situations than expected. However, in these animals there is the benefit of both spatial and temporal control of gene knockout. Despite the AAV serotype 9 showing neural trophism there is a chance of transfection of other cell types whereas the AdvCreERT2 is limited to *Advillin* expressing sensory and sympathetic neurons, giving tighter spatial control. Temporal control using tamoxifen allows the knockout to be timed to a specific developmental stage or point in an experiment i.e. before or after inducing a neuropathic pain injury using a model such as sciatic nerve ligation.

In this thesis we also did some pilot studies on the functional and behavioural impact of TrkA and p75 knockout in sensory neurons. Our *in vitro* experiments on TrkA or p75 knockout neurons (gene ablation via AAV9 Cre-eGFP IT injections *in vivo*) showed a decreased response to NGF in knockout cells, as measured by neurite outgrowth. This suggests that even in the adult system, despite neurons being independent of NGF for survival, they are responsive to neurotrophins, such as NGF, for regeneration of neurites following axotomy – as dissection of DRG for culture requires the cutting of peripheral terminals of sensory neurons. NGF secreted

in the inflammatory soup following inflammation or injury, has a role in both sensitisation of neurons but also in repair and axon guidance. It has been shown that in axotomy models, following the cut-off of retrograde transport of NGF from the periphery, apoptosis of neurons was observed supporting the role for NGF in survival of neurons (Levi-montalcini 1987). Therefore, decreased sensitivity to NGF *in vitro* following knockout of either the TrkA or p75 receptor may result in decreased repair and regeneration of neurons *in vivo* following axotomy. In our conditional knockout strains that we generated we found that contrary to studies using global knockout mice we observed no change in peripheral skin innervation of the hind-paw or changes in the cell size distribution in DRGs. Deficiencies in the peripheral nervous system in global knockout mice for either NGF or the NGF receptors result in hyposensitivity to mechanical and thermal stimuli, however, in both the *NTRK1* fl/fl; AdvCreERT2 and *NGFR* fl/fl; AdvCreERT2 animals there is no change in thermal sensitivity as measured using the static hot plate two weeks after induction of knockout via tamoxifen injections. A second cohort of *NTRK1* fl/fl; AdvCreERT2 animals was used to assess mechanical and thermal sensitivity four weeks after tamoxifen treatment however no change both tests was observed as measured using the static hot plate or Von Frey hairs respectively. Due to time constraints and the unavailability of animals the mechanical sensitivity of *NGFR* fl/fl; AdvCreERT2 animals was not assessed in this study. To fully characterise the effect of TrkA or p75 knockout more extensive behavioural testing and detailed investigation of anatomical changes needs to be performed as detailed below. To date, we did not see any gross changes in behaviour or anatomy in the animals tested. Overall, we have established and characterised in this study two methods for generating neuron targeted knockout of NGF receptors. This allows us to investigate the role of NGF in sensory neurons and to answer the question if NGF receptors have an essential role in pain and if they compensate for each other or function in synergy.

5.2 Future directions

We characterised MACS purified cultures and observed that despite a lag in neurite outgrowth after 24 hours, after 48 hours MACS purified neurons began to grow neurites. Treatment of neurons with conditioned medium taken from non-MACS purified cultures resulted in a significant increase in neurite outgrowth of MACS purified cells after 24 hours. In addition to NGF, conditional media from non-MACS purified cultures contains a number of other factors such as growth factors and members of the neurotrophin family such as BDNF and NT-3. To dissect the effect of NGF vs these other growth factors it is helpful to block NGF binding to TrkA, with a TrkA conjugated to IgG, and measuring the residual neurite outgrowth effect. Furthermore, the amount of other factors present in the conditional medium can be measured by ELISA. MACS purified cultures from wildtype mice or conditional knockout mice can be used in combination with the NGF inhibitors to further investigate changes in NGF signalling through the TrkA or the p75 receptor *in vitro*. As previously described activation of TrkA or p75 activate a number of different signalling pathways where TrkA signals via molecules such as MAPK, ERK and Ras, and $p75^{NTR}$ via JNK and c-Jun (Wilma J Friedman 2010; Bertrand et al. 2009), respectively. The complexity of NGF signalling has led to the hypothesis that the large degree of redundancy may result in different cell types preferentially using one downstream signalling pathways over another (Friedman and Greene, 1999). Therefore, using cultures from conditional knockout animals may provide further insight into the signalling pathways used following NGF signalling in pain. These experiments can be carried out following stimulation of cultures with both NGF and proNGF (the precursor to mature NGF). How pure neuron cultures alone respond to stimuli compared to non-MACS sorted cultures containing non-neuronal SGC will give an idea of how non-neuronal cells may modulate NGF signalling. This will be especially interesting as the targeted knockout of TrkA and $p75^{NTR}$ is localised to neurons and therefore

non-neuronal cells will retain expression of NGF receptors in non-MACS cultures such as TrkA on mast cells and $p75^{NTR}$ on SGCs.

MACS purification of DRG neurons has been shown to be effective in generating pure cultures of mainly small diameter neurons, which is a confound for studies investigating receptors or ion channels expressed on large diameter neurons. For the study of neurons expressing the TrkA or p75 receptors, MACS purification is useful as TrkA for example is expressed on the small diameter peptidergic C fibres. However, in cultures of small diameter neurons there is also the presence of the small diameter non-peptidergic C fibres. Therefore, in MACS purified cultures taken from *NTRK1* fl/fl or *NGFR* fl/fl animals injected IT with AAV9 Cre-eGFP a percentage of the GFP positive neurons might actually not be knockout for either the *NTRK1* or *NGFR* gene but could in fact be non-peptidergic neurons which do not express the gene of interest. Similarly, some of the control cells used might not express the gene of interest for the same reason. This could be avoided by counterstaining neurons against markers of non-peptidergic neurons such as IB4. It could also be useful in the future to further optimise the MACS purification protocol to isolate specifically p75 or TrkA expressing neurons. This would be achieved by running neurons thorough a second MACS column with a second batch of antibodies. In this way, the first step in the purification would be to use the non-neuronal antibody cocktail described in this study which tags all of the non-neuronal cells resulting in these cells being retained in the MACS LD column. A second sort using either antibodies against the p75 receptor or the TrkA receptor, or perhaps a combination of both, could then be used to tag all neurons expressing these receptors, which could then be sorted from the cell suspension and retained in the MACS column following a second sort. Of course, this protocol could not be used for generating MACS purified cultures following *NTRK1* or *NGFR* knockout either via AAV9 Cre-eGFP injection to deliver Cre or tamoxifen injection to activate Cre as these neurons would no longer express either the TrkA or p75 protein. However, using MACS

to specifically sort TrkA or p75 positive neurons from WT DRG could be used as an alternative method to confirm the effect of knocking out either NGF receptor in cultures of MACS purified DRG from either AAV9 or tamoxifen treated animals, through the use of specific receptor antagonists in these WT cultures. MACS sorting for p75 positive neurons has already been used as previously described, (chapter 2.4.1) (T.-V. V Nguyen et al. 2014) however, as this study has highlighted there have been issues optimising conditions for antibodies against the TrkA receptor however we identified a TrkA antibody that was suitable for staining TrkA in sections of L4 DRG. So, this antibody might be a candidate for such an approach.

As described in the previous chapter (chapter 3.4.4) MACS purified cultures isolated from *NTRK1* fl/fl and *NGFR* fl/fl animals injected with AAV9 Cre-eGFP could be further investigated for functional changes using calcium imaging and patch clamping. Detailed investigation using these methods can be performed by plating MACS purified cultures into microfluidic chambers to separate the cell body from the axons in different compartments. Using this technique, neurons are plated into a compartmental chamber where axons can grow across to a second chamber separated by microgrooves several days after plating. These microgrooves have a diameter large enough for axons to pass through however, they are too small for the cell bodies to pass through. Maintaining a higher volume of liquid on one side of the device allows movement of liquids from one compartment to the other by osmosis, creating effective separation of the two compartments (Tsantoulas et al. 2013). Using this setup separate treatments can be given to both the soma and axonal compartments, and the effect can be measured using both calcium imaging and patch clamping techniques. However, one confound of using the microfluidic technique with MACS purified cultures is that we discovered in this study that MACS purified neurons are difficult to adhere to either plastic plates or glass coverslips. In this study we use a thin coating of matrigel to aid adhesion of purified neurons to culture wells. However, compared to other substrates used to adhere cells, such as laminin,

matrigel forms a gel like layer and when used with the microfluidic chamber would prevent a tight seal being formed between the microfluidic chamber and the glass coverslip – which is critical in separating the compartments between the soma and axons. In addition to this, it is possible that matrigel could block the small microgrooves in the microfluidic chambers and retard the growth of axons from the soma chamber to the axonal chamber. As matrigel is a component of the extracellular matrix it has been used previously to resuspend cells before plating, thus allowing plating of cells within a matrix that is growth permissive (Bapat et al. 2016; Wyatt et al. 2011; Delree et al. 1989). Therefore, matrigel does not directly inhibit the growth of neurites in the dish or through the microgroove channels, however, it would prevent the passage of liquid though the channels. In order to direct the growth of neurites through the microgrooves, growth factors such as NGF are commonly added to the axonal compartment, matrigel blockage of the microgrooves would block the movement of liquids across the osmotic gradient in the microfluidic chamber, in addition to blocking the chemotractive signal by growth factors. This could be overcome by trialling the use of other adhesive compounds like fibronectin, which has not been tested as an adhesive for MACS purified neurons in this study. However, the need to use NGF or a similar substrate to direct neurite growth prior to experiments is another confound that despite benefits may make the use of microfluidic chambers unsuitable to investigate the effect of NGF following knockout of either the TrkA or the p75 receptor.

One method for validating findings from calcium imaging and patch clamping experiments using DRGs isolated from *NTRK1* fl/fl or *NGFR* fl/fl animals injected with AAV9 Cre-eGFP would be to repeated these experiments using DRGs isolated from *NTRK1* fl/fl; AdvCreERT2 or *NGFR* fl/fl; AdvCreERT2 Cre positive animals injected with tamoxifen.

In addition to *in vitro* experiments using both the AAV9 and tamoxifen mediated knockout of the NGF receptors, we would like to further investigate any changes in the anatomy of animals

following knockout. Using both models we noticed no gross anatomical changes, and confirmed knockout of the gene of interest at both the RNA and the protein level. Furthermore, Cre positive animals treated with tamoxifen showed no change in the cell size distribution of DRGs or innervation of the plantar skin of the hind-paw compared to global knockout studies suggesting that TrkA or p75 knockout in the adult mouse has no impact on survival of neurons. This could be confirmed by staining DRG sections for markers of different subpopulations of neurons including NF200 for large diameter neurons, IB4 for small diameter non-peptidergic C fibres and CGRP for peptidergic C fibres. Further to this other anatomical changes such as further investigation of fibres innervating peripheral skin and changes in the spinal cord could be performed. For example, in this study we did not investigate the effect of the knockout of p75 in the lumbar spinal cord in *NGFR* fl/fl; AdvCreERT2 Cre positive animals treated with tamoxifen. Another approach would be to confirm skin innervation and cell size distribution in tissue from *NTRK1* fl/fl or *NGFR* fl/fl mice injected IT with AAV9 Cre-eGFP.

It appears there is no change in expression of the complementary NGF receptors (i.e. no change in the expression of *NGFR* gene following targeted knockout of *NTRK1* gene or vice versa) as measured at the RNA level following knockout of either the TrkA or the p75 receptors except for *NGFR* fl/fl mice 2 weeks after AAV9 injection where a decrease in both *NTRK1* and *NGFR* gene expression was measured. However, the protein levels have not yet been investigated in regards to compensation of the complementary receptor. Therefore, one aspect to investigate in the future is to perform immunostaining and measure the percentage of neurons expressing p75 in *NTRK1* knockout mice and the percentage of TrkA expressing neurons in *NGFR* knockout animals using both AAV9 and tamoxifen mediated gene knockout to confirm there is no compensatory mechanism following knockout of one of the NGF receptors.

There are also interesting approaches to further investigate the functional and behavioural impact following knockout of either the TrkA or the p75 receptor to determine if one or both receptors are involved in NGF signalling in pain.

As already discussed (chapter 3.4.4), risk of spinal cord damage and variability in transduction following IT injection of AAV9 Cre-eGFP results in this model to be suboptimal for behavioural experiments. We therefore propose to use the generated conditional knockout strain for these experiments. Using this model has the benefit of temporal control where tamoxifen can be administered prior to, during or after behavioural testing. In combination with pain models this can be used to measure the impact of knockout of NGF signalling via either NGF receptor prior to pain, in the induction phase of pain, during the development of pain to investigate the role of NGF in the establishment and maintenance of peripheral or central sensitisation, or after pain to investigate a role for NGF in the resolution of pain. To fully characterise the effect following knockout of the TrkA or the p75 receptor, thermal sensitivity using the ramping hotplate or Hargreeves tail flick test to hot stimuli or testing using the cold plate or the acetone test to cold stimuli could be performed in addition to the tests that were performed in this study. Anxiety can be measured using the open field test which can also be used to investigate any motor impairments, in addition to the rotarod test to test for any off-target effects. Once any changes in the knockout animals have been identified it would be interesting to test these animals in inflammatory and neuropathic pain models. As described in chapter 1.4, preclinical models have been used where rodents injected with complete Freud's adjuvant (CFA) into the hind-paw, which induces inflammation as a model of inflammatory pain. In the naïve animal, following CFA treatment the resulting inflammation results in thermal sensitivity of these animals. However, following treatment with a TrkA-IgG or anti-NGF monoclonal antibody in CFA treated animals, no change in thermal sensitivity was observed suggesting that NGF signalling was required for the development of thermal pain in this model (Andreev et al.

1995; Lewin, Ritter, and Mendell 1993; Woolf et al. 1994; McMahon et al. 1995). We hypothesis that following knockout of either the TrkA or the p75 receptors we should see similar responses in inflammatory pain models, such as CFA treatment, where loss of NGF signalling stops the development of inflammatory pain.

While behavioural testing confirms the involvement of NGF in the development of pain – namely peripheral and central sensitisation – the downstream molecular changes in the neuron are unknown. Therefore, in addition to validating our findings from the RNA sequencing performed on *NTRK1* fl/fl; AdvCreERT2 Cre positive animals treated with tamoxifen, samples for RNA sequencing after induction of an inflammatory or neuropathic pain injury should also be collected to perform RNA sequencing to investigate changes in common downstream molecular signalling pathways. As mentioned previously, both the TrkA and the p75 receptors signal through several common signalling pathways in the cell, for example MAPK, ERK etc. (Kiris et al. 2014; Pezet and McMahon 2006; Capsoni et al. 2011; Capsoni and Cattaneo 2006; K. F. Lee et al. 1992). RNA sequencing could therefore be used to determine if NGF signalling in neurons is mediated by a particular pathway important for pain signalling. Once this is known it could be investigated whether knockout of one of the NGF receptors regulates this pathway or if a different pathway becomes active. This would also provide insight into how NGF signalling regulates downstream changes of known pain-related genes. For instance, it is known that NGF signalling results in rapid transcription of pain related genes such as VEGF, BDNF, TRPV1, substance P and CGRP, but RNA sequencing could highlight novel pain-related genes or signalling pathways previously overlooked (Pezet and McMahon 2006; Dubin and Patapoutian 2010; A. Patapoutian, Tate, and Woolf 2009). This in depth investigation might lead to the identification of novel pain-related genes, which could later be exploited as novel therapeutic targets.

In conclusion, we have characterised in this study two methods of specific knockout of TrkA and p75 in neurons which can be used to answer questions surrounding the role of NGF in pain. In addition to the pain models described above, these transgenic animals can be used for a number of different disease models. For example, NGF has been linked to arthritis which can be studied using the MIA model of arthritis (Rahman and Dickenson 2015; Sun, Beier, and Pest 2017). Diabetic polyneuropathy which can be studied using a diabetic mouse model such as the streptozotocin- induced diabetes (Shaqura et al. 2014) or investigating the role of p75 in suppressing angiogenesis of the retina as seen in diabetes (J. Zhang et al. 2014). Finally, NGF has a role in allergic airway hyperresponsiveness which can be studied using an allergic asthma model (Chen et al. 2014), in addition to studying neuroimmune interactions such as macrophage-neuron interactions. Furthermore, there is the potential to use the *NTRK1* fl/fl and *NGFR* fl/fl mouse lines in combination with other Cre lines in the future. Feil *et al*, describes a list of commercially available Cre mouse lines currently available, however few of them are CreERT2 and therefore tamoxifen controlled, but as the LoxP/Cre system is being more and more recognised as a powerful tool for creating transgenic mouse lines more conditional Cre lines will be produced with time (S. Feil, Valtcheva, and Feil 2009). It might be possible in the future to generate more inducible knockout mice for TrkA or p75 targeted to other cells of interest, for example astrocytes, oligodendrocytes or Schwann cells (S. Feil, Valtcheva, and Feil 2009). Similarly, by crossing the *NTRK1* fl/fl and *NGFR* fl/fl mouse lines with each other it is possible to generate a double knockout mouse to investigate complete knockout of NGF signalling.

Preclinical studies and phase II and III clinical trials relating pain and NGF have been highly effective in the reduction of pain. The use of the anti-NGF monoclonal antibody tanezumab in osteoarthritis for example has been shown to result in more than a 30% reduction in pain perception during walking. 30% reduction is determined as the clinical threshold for a

compound which produces a significant reduction in pain (Lane et al. 2010). Anti-NGF therapies are thought to be a potent novel therapy against pain which outperforms current pain treatments without having the harmful side effects that have been observed with current treatments, such as addiction to opioid treatments. However, as previously described (chapter 1.5) clinical trials with monoclonal anti-NGF antibodies potentially serious side effects, such as osteonecrosis, have been observed leading to a hold of these clinical trials from 2012 until 2016 when they recommenced after the potential causes of the side effects were uncovered. The FDA hold on anti-NGF (Hertz and Fields 2012; Holmes 2012) has delayed the progression of this novel treatment, however, the positive effects cannot be denied and it underpins a need for further investigation of NGF as a key player in pain. This study contributes to these investigations by developing a conditional and inducible knockout model for the TrkA and p75 receptors *in vivo* which can be continued to be utilised to investigate the role of NGF in pain.

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